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John William Coffin

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To my family

Abstract

Respiratory activity, folate levels and methionyl-tRNA transformylase activity were measured in mitochondria isolated from cotyledons of pea (*Pisum sativum* L. cv Homesteader) and peanut (*Arachis hypogaea* L. cv Early Spanish) during the first seven days of germination and growth. Rates of oxygen uptake of isolated mitochondria increased ca. 3-fold during the first 4 days in pea and peanut and continued to increase until the seventh day in peanut. Parallel trends were observed in respiratory control and ADP/O ratios.

Folate derivatives were extracted from whole cotyledons and isolated mitochondria. Differential microbiological assay using *Lactobacillus casei* (ATCC 7469) and *Pediococcus acidilactici* (ATCC 8081), combined with DEAE-celluose chromatography of mitochondrial derivatives, revealed the presence of a discrete pool of folates in these organelles of both species. The major derivatives in pea and peanut mitochondria were found to be polyglutamates of 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu.

Conditions for optimal assay of methionyl-tRNA transformylase were studied for both species. In pea mitochondria, transformylase activity rose rapidly to maximal levels after 4 days. This represented a 4-fold increase over the level at 18 hours. Transformylase activity reached maximal values after 3 days in peanut, and represented an increase of ca. 3-fold.

Cytochrome difference spectra and incorporation of radioactive leucine and methionine were examined in pea mitochondria. Organelles isolated after 3 days exhibited much higher levels of cytochromes 'b' and 'a+a_3' than mitochondria isolated after 18 hours. Conditions for optimal incorporation of amino acids were determined and the rates of incorporation measured in isolated mitochondria of two ages. Rates in 4-day organelles were found to be 5-fold higher than rates of 18 hour mitochondria. This incorporation was found to depend on an exogenous supply of ADP and malate. Contaminating bacteria, as measured by standard plate count assay, were 2×10^3 or less per assay, a level that would not contribute significantly to the rates of protein synthesis observed.

In both species, levels of mitochondrial folates, in particular those of 10-HCO-H_4PteGlu polyglutamates, closely paralleled changes in methionyl-tRNA transformylase and respiratory activity. The presence of protein synthesis in pea mitochondria, in conjunction with the above findings, points to a role of mitochondrial protein synthesis in the development of respiratory activity.

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List of Abbreviations

ATCC	American Type Culture Collection
BSA	bovine serum albumin
uCi	microCurie
CAP	chloramphenicol
CHI	cycloheximide
DEAE	diethylaminoethyl
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
PteGlu	pteroylglutamic acid (=folic acid)
H_4PteGlu	tetrahydropteroylglutamic acid
5-HCO-H_4PteGlu	N^5-formyl-H_4PteGlu
10-HCO-H_4PteGlu	N'^0-formyl-H_4PteGlu
5,10-CH=H_4PteGlu	N^5,N'^0-methenyl-H_4PteGlu
5,10-CH_2-H_4PteGlu	N^5,N'^0-methylene-H_4PteGlu
5-CH_3-H_4PteGlu	N^5-methyl-H_4PteGlu
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
PPO	2,5-diphenyloxazole
POPOP	1,4-di[2-(5-phenyloxazolyl)]-benzene
S.E.M.	standard error of the mean
TES	2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-amine}ethanesulfonic acid
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane

I. INTRODUCTION

Germination and early growth of seeds are extremely complex phases of development and have therefore attracted a considerable amount of study. These periods of dynamic change involve the interaction of numerous factors and thus research has evolved along several lines. Areas investigated include seed maturation as well as aspects of dormancy. Several workers have also examined the metabolic processes occurring during this time, particularly respiration and the mobilization of storage reserves. Others have studied the role of plant growth regulators and other factors involved in the control of the above events. In addition, the importance of seedling establishment to the success of a species has prompted research on germination and early growth from an ecological, as well as an agricultural perspective.

The Introduction of this thesis will focus on certain aspects of the biochemistry of germination and early growth of seeds with particular reference to the development of a competent respiratory system. The research presented in this thesis was performed using two legume species, *Pisum sativum* (pea) and *Arachis hypogaea* (peanut). Several aspects of germination and growth have been studied in numerous other species. However, due to the extensive nature of these studies, the literature discussed in this section, will be confined, where possible to previous work on pea and peanut. The reader's attention is directed to many excellent books

and review articles which cover portions of the subject of germination not dealt with in detail in this thesis. Books giving this topic overall coverage include those written by, Bewley and Black (1978, 1982, 1985), Khan (1977 and 1982), Kozlowski (1972) and Mayer and Poljakoff-Mayber (1982). Recent articles have reviewed such areas as seed development (Dure, 1975; Higgins, 1984; Millerd, 1975), dormancy (Taylorson and Hendricks, 1977; Khan, 1980/81), reserve mobilization (Ashton, 1976; Chrispeels et al., 1979) and control of germination (Mayer, 1980/81; Mayer and Shain, 1974; Wareing and Saunders, 1971). Agricultural aspects have been examined by Arora (1983) and by Matthews (1977).

The Introduction of this thesis is divided into several sections. The first is concerned with the nature of reserves found in seeds of pea and peanut, in addition to the mobilization of these reserves, with an emphasis on the timing of this process. The second section examines respiratory activity of these seeds and relationships between this activity and reserve mobilization. Subsequent sections consider mechanisms potentially involved in the development of respiratory activity, particularly mitochondrial biogenesis and mitochondrial protein synthesis. The role of folate metabolism in these latter two processes is also discussed.

Mobilization of Stored Reserves

Two principal characteristics of seeds are the presence of stored reserves and the enzyme systems necessary to convert these reserves to a form useable by the growing seedling. The major storage products are carbohydrates (sugar or starch), proteins and fats. Organic phosphates and some inorganic compounds are also stored in the mature seed as phytin, the mixed Ca^{2+}, Mg^{2+} and K^{+} salts of myoinositol hexaphosphoric acid (Bewley and Black, 1978). Storage reserves, synthesized during seed maturation, are hydrolyzed during the early stages of growth and nutritionally support the seedling until it becomes photoautotrophic. Although plants store all three major types, two usually predominate. The relative quantities of each is species-dependent. For example, pea seeds contain, as a percentage of dry weight, ca. 25% protein, 52% carbohydrate and 6% fat whereas peanut contains ca. 31% protein, 12% carbohydrate and 48% fat (Bewley and Black, 1978).

Carbohydrate reserves, comprised mainly of starch, are stored intracellularly as starch grains. Protein is found in organelles named protein bodies and fats may exist as droplets in the cytoplasm, but probably more often in discrete organelles termed oil bodies or sphaerosomes (Esau, 1977). The majority of storage products are found either in endosperm tissue or as a part of the embryo itself, in the cotyledons.

Hydrolysis of stored reserves has been examined in a number of species (for reviews see Bewley and Black, 1978 and Mayer and Poljakoff-Mayber, 1982). Although differences exist between species, some generalizations can be made. In most species examined, degradation of reserves begins shortly after imbibition and is often biphasic. That is, initially hydrolysis occurs slowly, but after a few days a marked increase in activity is observed. In the following discussion, the time of occurrence of specific events is given in hours or days after the beginning of imbibition. That is, the seed soaking period is included as part of the experimental time period.

The chief storage proteins of legumes are the globulins. In pea, for example, they comprise 75-80% of total seed protein. On the basis of physical criteria, globulins have been divided into two large groups; the legumins and vicilins. Within a species, some heterogeneity exists in either type among cultivars, but between species there is even greater variation. In pea, the principal storage proteins are legumin and vicilin. The analagous proteins in peanut are arachin and conarachin. Details on the protein content, types and structure are given in a recent article (Mosse and Pernollet, 1983), and in the text of Daussant et al.(1983).

Hydrolysis of protein reserves has been studied in a number of legumes. Konopska (1978;1979), using SDS gel electrophoresis, examined the changes in levels of the two

major storage proteins of *P. sativum* cv. Lagiewbicki, isolated from etiolated tissue during a 5 day period. Protein began to be degraded after three days, but the most rapid decrease began on the fifth day. This same result was found by Basha and Beevers (1975) studying the Burpeeana variety of pea. These workers also examined the ability of cotyledon extracts to hydrolyze radioactively-labelled vicillin and legumin. As expected from the electrophoresis data, the most rapid hydrolysis occurred after 5 days. Earlier studies by Larson and Beevers (1965), on amino acid metabolism in Alaska pea seedlings, showed an almost ten-fold increase in shoot amino acid levels in the period from the third to the tenth day. The most rapid increase occurred after 5 days. Cotyledon levels rose only slightly, indicating that the bulk of the amino acids were transported to the shoot. Protein hydrolysis in light-grown Alaska pea however, occurs in a linear fashion, rather than biphasic (Guardiola and Sutcliffe, 1971). Whether this difference is due to growth conditions or to varietal differences is not clear.

Protein hydrolysis has also been studied in peanut. The biphasic nature of degradation present in pea is also found in this species, with the period of most rapid hydrolysis occurring after 4 days (Basha and Cherry, 1978). The activities of protein-degrading enzymes in other species have been recently reviewed (Mikola, 1983). Biphasic protein degradation has recently been shown to occur in *Phaseolus*

vulgaris (Nielsen and Liener, 1984).

Starch reserves are comprised of two types of glucose polymers; amylose and amylopectin. Amylose consists of glucose residues joined by alpha-1,4-linkages, whereas amylopectin contains alpha-1,4- as well as some alpha-1,6-linkages that cause branching in the molecule (Salisbury and Ross, 1978). Starch is degraded by a series of enzymes and is accomplished by hydrolysis as well as phosphorolysis. Hydrolytic enzymes include alpha- and beta-amylase, alpha-glucosidase and debranching enzymes. The final product of the activity of these enzymes is free glucose, which can be converted to sucrose, the form of carbohydrate transported to the seedling. Glucose-1-phosphate, produced by phosphorylase, is also converted to sucrose.

Mobilization of starch reserves has been examined in pea and the pattern is similar to that of protein hydrolysis. Degradation is often biphasic, although in at least one cultivar, Victory Freezer, it is linear (Bain and Mercer, 1966). Juliano and Varner (1969) studied amylase activity in the Early Alaska variety of pea. The activity of both alpha- and beta-amylase increased markedly after 8 days following imbibition. These authors also examined phosphorylase levels and found that activity rose rapidly after 3 days to peak at 5 days. Levels of starch did not decrease rapidly until after 7 days, suggesting that the major pathway of starch degradation is via hydrolysis rather than phosphorolysis. Levels of free sugars did not rise in

the cotyledons, implying a rapid transport of these compounds to the seedling (Juliano and Varner, 1969). Yomo and Varner (1973), in a study of amylase activity in the Alaska pea, showed that alpha- and beta-amylases began to increase after 4 days and to peak at 10 days. Although little work has been done on carbohydrate degradation in peanut, most likely due to the low levels of this class of compounds, it is probable that a process analogous to that of other seeds, occurs in this species. A recent study has also examined alpha-amylase levels in barley (MacGregor et al., 1984).

Oil reserves are degraded in a stepwise manner. These reserves, present mainly as triglycerides, are first converted to glycerol and fatty acids by the action of lipases. Beta-oxidation in the glyoxysomes converts fatty acids to acetyl-CoA, which then enters the glyoxylate cycle, where in every turn of the cycle, a molecule of succinate is formed. This compound is converted to oxaloacetate by the Krebs cycle enzymes in the mitochondria, and subsequently to phosphoenolpyruvate (PEP). Sucrose is synthesized from PEP by a combination of gluconeogenesis and sucrose formation.

The disappearance of oil reserves is usually accompanied by an increase in the number of glyoxysomes (Beevers, 1979). This increase is paralleled by the activity of the two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase. Although the most complete work on this process has been performed using castor bean (for a

review, see Beevers, 1979), several studies have been carried out on peanut. Longo and Longo (1970), using a combination of ultrastructural and biochemical techniques, showed that glyoxysome biogenesis was correlated with the development of isocitrate lyase and malate synthetase activity. Huang (1975) confirmed the presence of glyoxysomes in peanut and gave evidence that these organelles were similar in structure to those of other species. Synthesis *de novo* of the two key enzymes was shown by two research groups (Longo, 1968; Gientka-Rychter and Cherry, 1968). In peanut, the largest increase in isocitrate lyase and malate synthetase activity occurred after 3 days (Longo and Longo, 1970; Marcus and Velasco, 1960). No activity was detected by these workers in the first 24 hours. Glyoxysomes and their associated enzyme activities disappear after lipid degradation is complete (Beevers, 1979). Alkaline lipase activity in peanut cotyledons was shown to increase ca. 4-fold from the third to fourth day after the onset of imbibition (Huang and Moreau, 1978).

It is clear from the preceding paragraphs that the germination and early growth of pea and peanut seeds is characterized by a marked increase in metabolic activity. In most species, this occurs about 3 to 4 days from the start of imbibition. Although a part of the products of reserve breakdown would conceivably be used in synthetic reactions within the cotyledons, the bulk are transported to the seedling.

Respiratory Activity of Seeds

Perhaps the most easily observable aspect of the increased metabolic rate of germinating seeds is the rise in respiratory activity of whole seeds and of mitochondria isolated from these seeds. Dry seeds are characterized by extremely low levels of respiratory activity. Given appropriate conditions of water, temperature and atmosphere, there is a marked increase in oxygen uptake (Mayer and Marbach, 1981). Respiration has been examined in a large number of species including peanut and several cultivars of pea, and this work, in addition to studies in other species, has been recently reviewed (Bewley and Black, 1985; Mayer and Poljakoff-Mayber, 1982).

Germination and early growth of seeds are clearly energy-requiring processes. In this regard, metabolic energy is required for several reactions involved in reserve mobilization. For example, ATP is essential for certain steps in sucrose synthesis, beta-oxidation of fatty acids and gluconeogenesis. Other processes such as protein synthesis are also ATP-dependent.

Adenosine triphosphate can be generated in seeds in at least two ways; by alcohol or lactic acid fermentation and by oxidative phosphorylation. Fermentation, normally functioning under anaerobic conditions, produces only 2 ATPs per molecule of glucose metabolized, in addition to ethanol or lactic acid. Oxidative phosphorylation, occurring under aerobic conditions, produces 36 ATPs and CO_2. The two

systems have been shown to be present in a number of seeds during germination and growth, although the relative contribution of each pathway, has not been as extensively evaluated (Pradet, 1982).

One of the chief difficulties encountered in a discussion of respiratory activity during germination and growth of seeds is that studies of this process have been performed using different species, in addition to different cultivars of the same species. This makes direct comparison difficult and perhaps even incorrect. A few examples will illustrate this point. Marbach and Mayer (1976) showed differences in respiratory activity and reserve mobilization in two species of pea, *P. sativum* and *P. elatius*. Mitochondrial activity persisted longer and reserve degradation occurred later in *P. elatius*. Kolloffel (1967), and Nakayama et al. (1978), studied O_2 uptake in mitochondria isolated from two cultivars of *P. sativum*; Rondo and Alaska, respectively. These authors demonstrated that O_2 uptake decreased rapidly from maximum rates, after 2 days. These findings are in contrast to Solomos et al. (1972) who studied mitochondria isolated from the Homesteader cultivar, and Bain and Mercer (1966) who measured, manometrically, O_2 uptake of whole cotyledons of the Victory Freezer cultivar. These latter workers showed that O_2 uptake rose from initially low values to maximal rates at 4 days.

It is possible that the differences noted above may be related to the cultivar of the plant material. Certain

physiological or biochemical processes present in one plant cultivar have been shown to markedly different in another. For example, alpha-amylase exhibits large variations in activity among barley cultivars (MacGregor, 1978). In addition, however, different growth conditions may have a pronounced effect on the results obtained. For example, it is known that imbibition temperature can affect the rate of water uptake (Nawa and Asahi, 1973a). In the Alaska pea, root length after 9 days at 19°C is half that obtained when seedlings were grown at 23°(Young et al., 1960). ATPase actvity, measured by these authors, also exhibited marked temperature effects. At 16°, activity was low and did not increase during the 9 day period. Growth at a temperature of 19° produced a five-fold increase in activity over the same period although no maximum level was reached. An eight-fold increase over the 16° values, was shown by plants grown at 23°, with a sharp optimum occurring at 5 days. These results clearly show that large differences can result from small changes in temperature.

Enzyme assay and organelle isolation techniques employed by workers studying germination and growth, also vary and may be a factor in the generation of the observed differences. Some of the research outlined above was performed over fifteen years ago and it is well known that more recent techniques of organelle isolation have yielded improvements in organelle quality (Quail, 1979). With these concerns in mind, however, overall trends can still be

identified. It is clear that during imbibition and at least shortly thereafter, O_2 uptake rises rapidly. The precise timing and persistence of this respiratory activity is variable.

Several studies of mitochondrial respiration have been performed using the pea cultivar, Alaska. Oxygen uptake increases rapidly upon imbibition and reaches maximal levels after 24 hours (Nawa and Asahi, 1971; Nawa and Asahi, 1973a). This may be maintained for at least another 48 hours (Nawa and Asahi, 1971; Morohashi, 1980), although Nakayama et al. (1978) have shown a sharp decrease to 40% of the maximum, after 2 days. Nawa et al. (1973) indicated that there was a much slower decline, with a decrease to only 75% of the maximum by 6 days, although RC and ADP:O ratios dropped markedly after the second day. These variations are somewhat surprising since these studies used the same cultivar and virtually the same techniques. Regardless of the differences, however, it is clear that, in this cultivar, oxygen uptake rises quickly after the start of imbibition to a maximum at 1-2 days. Subsequently, mitochondrial integrity, as measured by RC and ADP:O ratios, begins to be lost.

The trends observed by these authors are in agreement with Morohashi and Bewley (1980), who also examined respiratory activity during the first 24 hours after the onset of imbibition. Oxygen uptake coupled to ADP phosphorylation could be observed when mitochondria were

isolated from cotyledons after two hours imbibition and were supplied with succinate or NADH. Malate and alpha-ketoglutarate were not effective substrates until after at least twelve hours. These results suggest that the mitochondrial enzyme systems were incomplete at the very early stages of imbibition.

The activities of several key mitochondrial enzymes have also been examined. Cytochrome c oxidase, malate dehydrogenase and succinate dehydrogenase increase in parallel with rising oxygen uptake levels (Nawa and Asahi, 1971; Nawa et al., 1973). Recently, Asahi's group, using an immunological approach, has demonstrated the presence of two subunits of cytochrome c oxidase in mitochondria isolated from dry seeds and during the early stages of imbibition. These authors have suggested that these and other subunits are present in a free form in the dry seed, are reassembled during seed hydration and that this reassembly results in the observed increases in cytochrome c oxidase activity (Matsuoka et al., 1981; Matsuoka and Asahi, 1983). Research concerning oxidative phosphorylation in these and other species, during the early stages of germination, has recently been reviewed (Pradet, 1982).

As noted in the previous section, reserve mobilization has been studied in the Alaska cultivar. Cotyledon protein levels began to drop after the second day and reached ca. 30% of the original level by 6 days (Larson and Beevers, 1965; Guardiola and Sutcliffe, 1971). Since respiratory

activity reaches a maximum at 24-48 hours, there is good correlation between periods of rapid reserve breakdown and high mitochondrial activity. There appears to be, however, no correlation between maximal respiratory activity and maximal activity of the degradative enzymes. For example, amylase activity rose rapidly after 8 days to peak at 9 days (Juliano and Varner, 1969), although Shain and Mayer (1968) showed an increase after 3 days. Proteolytic activity increases sharply after 7 days and remains high for several more days (Yomo and Varner, 1973). It may be expected that, due to the energy requirements of mobilization, high mitochondrial and degradative enzyme activity would occur concurrently. Maximum beta-mannanase activity has also been shown to occur after most of its substrate, galactomannan, has disappeared. The reason for this apparent anomaly was not explained. (McCleary and Matheson, 1975 and 1976).

Mitochondrial respiratory activity has also been studied in the Homesteader cultivar (Solomos et al., 1972). Although coupled mitochondria could be detected after 24 hours, maximal rates of oxygen uptake did not occur until 4 days. Respiratory control ratios also increased from 3.5 to 4.4 between the third and fourth days. In addition, succinic dehydrogenase and cytochrome c oxidase activities exhibited maximal activities by 4 days (Solomos et al., 1972). These results indicate that mitochondria are not fully active until that time, and are therefore in contrast to studies of the Alaska pea, where maximum rates occurred earlier. The

differences noted suggest that the two cultivars are indeed quite different and comparing the timing of events in the two cultivars may therefore be invalid, although comparison of trends would not.

Reserve mobilization, to this author's knowledge has not been studied in the Homesteader cultivar, although it is conceivable that reserve degradation in this cultivar may be biphasic, with the period of most rapid decrease occurring after 4-5 days. Protein degradation in the Burpeeana cultivar follows this pattern (Basha and Beevers, 1975). In Homesteader, rates of oxygen uptake remained high at least until 8 days, mitochondria retained a high degree of coupling (Solomos et al., 1972) and would presumably be capable of supplying the necessary energy for mobilization.

There is also evidence that, in some seeds, fermentation may supply at least some of the energy requirements. Ethanol is known to accumulate in seeds during germination and shortly thereafter. This compound, produced by a conventional glycolytic pathway, is subsequently metabolized. (Cameron and Cossins, 1967; Cossins and Turner, 1962). Ethanol metabolism in plants has been the subject of a recent review (Cossins, 1978).

In the Homesteader cultivar, alcohol dehydrogenase activity rises almost two-fold from initially low values, to a peak at 2 days, and subsequently decreases (Cossins et al., 1968). This enzyme has also been shown to be present in the Alaska cultivar (Suzuki and Kyuma, 1972). Activity

increases to a maximum at 2 days but is almost non-existent at 4 days. Ethanol accumulation has also been examined in several other species including corn, wheat, barley, lentil and several bean cultivars (Cossins and Turner, 1963; Leblova et al., 1974; Morohashi and Shimokoriyama, 1975a).

Lactic acid accumulation, in addition to ethanol, has been demonstrated in pea seedlings (Cossins, 1964; Cossins and Turner, 1963; Leblova et al., 1974). Lactate dehydrogenase reaches maximum levels at 1 day and declines to half this activity by 2 days (Leblova et al., 1974). Lactate levels were lower, by an order of magnitude, than ethanol levels (Leblova et al., 1974), and disappeared prior to the decrease in ethanol levels (Cossins and Turner, 1963; Leblova et al., 1974).

The high activities of lactate and alcohol dehydrogenases during early seedling growth strongly suggest that aerobic respiration is functioning at low levels since accumulation of ethanol and lactic acid would not be expected under aerobic conditions. It has been suggested that the anaerobic conditions apparently existing at these early stages, may be a result of the testa acting as a barrier to oxygen diffusion since ethanol levels drop markedly after radicle protrusion (Cossins, 1978; Suzuki and Kyuma, 1972). A physical barrier may, however, be only partially responsible for the presence of high levels of fermentation. When pea seeds were germinated and grown with and without the testa, it was demonstrated that absence of

the testa resulted in only a slight increase in oxygen uptake, even when the seeds were placed in an atmosphere of 100% oxygen (Kolloffel, 1967). Solomos et al. (1972) examined CO_2 output and respiratory quotients (RQ) of pea cotyledon slices prepared over an eight-day germination period. The high RQ and rates of CO_2 output noted by these authors during the first 2 days (even when the thin slices used should have allowed adequate oxygen diffusion) further support the contention that a barrier is only partially the cause. The RQ declined sharply from a value of 1.3 to 0.85 between the second and third days. The output of CO_2 exhibited a decrease after the third day. Rates of oxygen uptake rose rapidly from 1 day to a maximum at 4 days. These trends were also observed by Spragg and Yemm (1959). The results of these studies strongly suggest that mitochondria were not fully active initially, but became active after 3-4 days. It is conceivable that mitochondria at these early stages have a reduced capacity to utilize oxygen and therefore, fermentative pathways play a major role in supplying the energy demands of the cotyledon. This contention is supported by the results of Morohashi and Shimokoriyama (1975a and b) who studied the relative activities of glycolytic and mitochondrial pathways during germination and early growth of *Phaseolus mungo* seeds. These authors concluded that initially, alcohol fermentation was more important than oxidative phosphorylation, since the former pathway was an order of magnitude more active than the

latter.

The occurrence of fermentation during the first few days is likely to be at least partially responsible for the biphasic nature of reserve breakdown noted for some seeds. Fermentation supplies only a fraction of the ATP generated by oxidative phosphorylation and therefore maximum rates of reserve degradation can only occur when mitochondria are fully active.

Development of mitochondria has been examined from an ultrastructural perspective. The results support the biochemical evidence that these organelles are lacking some components at the earliest stages of germination and growth. In species studied to date, mitochondria initially lack distinct cristae, but subsequently fully-developed organelles appear. The time for this development varies between cultivars of pea. Bain and Mercer (1966), in a study of the Victory Freezer cultivar, showed that fully-developed mitochondria were evident by 2 days, although maximum oxygen uptake did not occur until 5 days. Mitochondria of the Homesteader pea are well-developed by 4 days (Solomos et al., 1972). Cotyledons of the Alaska cultivar exhibit structurally complete mitochondria after only 18 hours (Nawa and Asahi, 1973b).

Although fewer studies have been performed using peanut, biochemical and structural evidence strongly suggests there are many similarities to the pattern of mitochondrial development in pea. Oxygen uptake rates increase

rapidly until at least 5 days and the activities of succinate and malate dehydrogenases, cytochrome c oxidase and NADH-cytochrome c reductase parallel this increase (Morohashi et al., 1981a). Ultrastructural evidence has demonstrated that, similar to the situation in pea, peanut mitochondria are initially poorly developed but attain normal structure by 4 days (Morohashi et al., 1981a and b). Earlier studies in peanut demonstrated a correlation between increases in mitochondrial respiratory activity and mitochondrial protein levels during germination and growth (Breidenbach et al., 1966). In a subsequent paper, the amount of DNA obtained from isolated mitochondria was also shown to increase during this time (Breidenbach et al., 1967). These authors concluded that biogenesis of mitochondria was occurring in the cotyledons. Cherry (1963), in an examination of enzyme changes in peanut, presented evidence for a close relationship between high mitochondrial activity, reserve breakdown and degradative enzyme activity. A good correlation between mitochondrial activity and reserve mobilization has also been shown in castor bean (Beevers, 1979). The number of glyoxysomes and the activity of key glyoxylate cycle enzymes rose rapidly between 2 and 4 days in parallel with increasing levels of oxygen uptake (Akazawa and Beevers, 1957; Gerhardt and Beevers, 1970).

It has been proposed that the patterns of mitochondrial development in pea and peanut are basically different (Bewley and Black,1978). Increases in respiratory activity

in pea do not appear to require mitochondrial protein synthesis (Nawa and Asahi, 1973b; Malhotra et al., 1973; Solomos et al., 1972). The opposite appears to be true for peanut (Morohashi et al., 1981a and b). The absence of protein synthesis in pea mitochondria implies that mitochondrial biogenesis is not occurring, since biogenesis requires input from the mitochondrial translation system (Schatz and Mason, 1974). Therefore, it is possible that increases in mitochondrial activity in peanut are due to increases in the number of these organelles through biogenesis, whereas in pea, mitochondrial development occurs via improvement of pre-existing organelles (Bewley and Black, 1978). The next section discusses some aspects of the biogenesis of mitochondria, focussing on the mitochondrial translation system and relationships between protein synthesis and the development of respiratory activity.

Biogenesis of Mitochondria

Mitochondria of all species examined to date, contain DNA molecules unique to these organelles. The primary function of the mitochondrial genome is biogenesis of these organelles (Tzagoloff, 1982). During cell division, the numbers of mitochondria are increased by simple division of the organelles, thus enabling a constant population to be maintained (Luck, 1965). The ability of mitochondria to fuse and divide has been observed in living tissue using the technique of phase-contrast microscopy (Wildman et al.,

1962). Maturation of recently-divided mitochondria, ie growth in mass, is accomplished by incorporation of newly-synthesized material into these organelles. Synthesis *de novo* of mitochondria from macromolecular precursors has not been demonstrated and is considered to be very unlikely (Tzagoloff, 1982). Proteins present in many organisms are known to turn over during the life of cells (Goldberg and Dice, 1974; Huffaker and Peterson, 1974) and this also appears to be the case for mitochondria (Tzagoloff, 1982). Therefore, biogenesis of mitochondria involves protein turnover and increases in mass, in addition to the regulation of mitochondrial number.

Mitochondrial DNA codes for a small, but essential number of proteins, and the rRNAs and tRNAs necessary for the translation of organelle proteins. The importance of the mitochondrial genome is illustrated by studies of yeast (*Saccharomyces cerevisiae*), where deletion of the entire mitochondrial genome, or even portions of it, results in the absence of functional mitochondria. These mutants, known as petites due to their small colony size, can only grow on fermentable substrates. Studies of yeast exhibiting the petite phenotype and its non-Mendelian inheritance, by Boris Ephrussi in the late 1940's and early '50's, formed the basis of mitochondrial genetics and provided special impetus for further research in this field (Ephrussi et al., 1949a and b).

Comparative studies of mitochondrial DNA has shown the mitochondrial genome to be functionally conservative. That is, basically the same genes are present in all species examined, although not all genomes have been studied in the same detail (Gray, 1982). The majority of research on the mitochondrial genome has been performed using yeast and *Neurospora crassa*. The many available mutants of these species, in addition to the ability of yeast to survive without functional mitochondria, has facilitated this research. The complete sequence of the bovine (Anderson et al., 1982), and human mitochondrial genomes are known and has greatly increased the understanding of mitochondrial genome organization (Anderson et al., 1981).

Although the mitochondrial genome is functionally conservative, there is a great variation in its organization among the major groups of eukaryotes. Mammalian genomes have, so far, been demonstrated to be the smallest (ca. 17 kilobase pairs, Kb) and the most compact (Anderson et al., 1981). There is virtually no intergenic DNA and no split genes. The tRNA genes separate nearly all individual rRNA and structural genes and may function as recognition signals in RNA processing (Attardi, 1981a and b). In contrast to mammalian genomes, yeast and *N. crassa* mitochondrial genomes are much larger (ca. 78 and 60 Kb, resp.) and contain some split genes in addition to large A+T-rich regions. The gene products coded for by yeast mitochondrial DNA, however, appear to be similar in number and type to mammals (Gray,

1982). The mitochondrial DNA of higher plants represents the largest organelle genomes known, in addition to being the most variable (Levings, 1983). The largest plant mitochondrial genome is 160 times the size of the human mitochondrial genome, and there is even a seven-fold size variation in one family, the Cucurbitaceae (Ward et al., 1981; Bendich, 1982). As no complete sequences exist for plant mitochondrial genomes, some of the variation may be due to variation in the results obtained by the techniques used to study genome size and complexity (Edelman, 1981). Plant mitochondrial genomes remain the most poorly understood and research has lagged behind that of chloroplast genomes (Leaver et al., 1982). Studies of the organization and expression of the chloroplast genome have been the subject of recent reviews (Bedbrook and Kolodner, 1979; Ellis, 1981). Detailed information concerning organelle genomes of other species can be found in Borst et al.,(1984); Gillham (1978), Gray (1982), Kroon and Saccone (1980), Tzagoloff (1982) and Yaffe and Schatz (1984).

RNAs coded by mitochondrial genomes are distinct from their cytoplasmic counterparts. In plants, organelle rRNAs more closely resemble prokaryotic rRNAs than eukaryotic ones. In wheat mitochondria for example, fingerprint analysis of the 26S, 18S and 5S rRNAs demonstrated that the sequence of these molecules was markedly different from the cytosolic ribosomal subunit sequences (Cunningham et at., 1976). Further work from this laboratory revealed that, on

the basis of sequence homology to *Escherichia coli* 16S rRNA, mitochondrial 18S rRNA was clearly prokaryotic in nature (Bonen et al., 1977). The sequence of the 5S rRNA, however, revealed it was not closely homologous to either prokaryotic or eukaryotic cytosolic 5S RNA and that it contained some unique features (Spencer et al., 1981). It is interesting to note that the presence of 5S rRNA in mitochondria is unique to higher plants.

Other aspects of the mitochondrial translation system are also unique to these organelles. The mitochondrial tRNA molecules can be separated from cytosolic tRNAs, as well as chloroplast tRNAs, using electrophoretic and chromatographic separations (Edelman, 1981). Mitochondria also contain a discrete set of aminoacyl-tRNA synthetases (Lea and Norris, 1977). The genetic code read by mitochondrial tRNA is different from the cytosol. For example, the triplet codon UGA, normally read as a 'stop' codon, is read as tryptophan in animal and fungal mitochondria (Lagerkvist, 1981). Fewer tRNAs are required for translation in mitochondria due to an expanded codon recognition pattern. That is, a single tRNA species can recognize all of the codons of a four-codon family specifying a single amino acid (Lagerkvist, 1981; Fox, 1981).

Although there are several similarities between mitochondrial and prokaryotic genome organization and translation systems, distinct differences also exist. Thus, the organelle system is unique. The possible significance of

these similarities and differences has been the subject of much discussion with respect to the evolutionary origins of these organelles. Recent reviews of this subject have appeared in the literature and include those by Gray (1982), Gray et al. (1984), Gray and Doolittle (1982), Doolittle (1982) and Margulis (1981). The evolutionary origin of mitochondria and chloroplasts continues to be a topic of lively debate.

As noted above, mitochondria synthesize only a small number of their own proteins. The remainder are coded by nuclear genes and are synthesized in the cytosol. Most of the research concerned with identification of organelle-synthesized proteins has been done using yeast and *N. crassa*. In these species, ca. 10-12 polypeptides are synthesized. In general, proteins encoded by the mitochondrial genome tend to be very hydrophobic in nature and function in electron transport and oxidative phosphorylation. The proteins so far identified are subunits of cytochrome c oxidase and oligomycin-sensitive ATPase, the protein moiety of cytochrome b, in addition to a protein named var 1 (Tzagoloff et al., 1979). Evidence is accumulating that these proteins, with some variations, are made in all mitochondria (Leaver and Gray, 1982). Two of the polypeptides synthesized by isolated plant mitochondria, have been tentatively identified as subunits of cytochrome c oxidase (Leaver et al., 1982). The remaining unidentified proteins are likely to be similar to those found in fungal

mitochondria, although there is a possibility that higher plant mitochondria synthesize a greater number of proteins than organelle systems examined to date (Leaver and Gray, 1982).

Mitochondrial protein synthesis is sensitive to inhibitors of prokaryotic protein synthesis such as chloramphenicol and erythromycin, and is insensitive to inhibitors of oytoplasmic protein synthesis (Schatz and Mason, 1974). The difference in sensitivity to these inhibitors has been exploited in determining the site of synthesis of several mitochondrial proteins. For example, in *in vivo* experiments, proteins synthesized in the presence of cycloheximide, but not in chloramphenicol, are said to be synthesized in mitochondria. Studies of this nature have been used to great advantage in yeast and *Neurospora* (Schatz and Mason, 1974).

As stated at the beginning of this section, the primary function of the mitochondrial genome is organelle biogenesis. This is accomplished via the mitochondrial translation system which consists of all the necessary components contained within the organelle. The obvious importance of the mitochondrion to cell function has prompted research into processes involved in the proliferation of this organelle. Biogenesis has been studied in several unrelated species. Chloramphenicol has been shown to sharply decrease mitochondrial protein synthesis in the flight muscle of newly-emerged blowflies (Ashour et al.,

1980a and b; Ashour and Tribe, 1981). Later stages in the development of the flies exhibited lower rates of mitochondrial protein synthesis and were less sensitive to the inhibitor. Ultrastructural studies by these authors revealed that mitochondria from chloramphenicol-treated flies were abnormal and showed poorly-developed internal structure. The results strongly suggest that biogenesis of mitochondria occurs during early development of blowflies.

Fungal spore germination, similar to germination of seeds, is characterized by a large increase in oxygen uptake. Brambl's group has examined the role of mitochondrial biogenesis in the development of respiratory activity in two fungal species. It was shown in *N. crassa* and *Botryodiplodia theobromae* that cytoplasmic protein synthesis was required for increases in respiratory activity during the early stages of spore germination, whereas mitochondrial protein synthesis was not. This latter process, however, was required at later stages; ie, after germ tube emergence (Brambl, 1980; Stade and Brambl, 1981; Wenzler and Brambl, 1981). Inhibition of mitochondrial protein synthesis in rat liver by oxytetracycline demonstrated that this treatment resulted in impairment of liver regeneration of partially-hepatectomized rats, further illustrating the importance of mitochondrial protein synthesis in cells (van den Bogert et al., 1983).

As outlined in the previous section, the role of mitochondrial biogenesis in the development of respiratory

activity has been examined in plants (Malhotra and Spencer, 1973; Malhotra et al., 1973; Morohashi et at., 1981a and b; Nawa and Asahi, 1973b). These studies also utilized the different sensitivities of the mitochondrial and cytosolic translation systems to inhibitors of protein synthesis. This formed the basis for the proposal that mitochondrial protein synthesis is required in peanut, but not in pea, during germination and early growth (Bewley and Black, 1978). Biogenesis of plant mitochondria has been shown to occur in response to wounding (Sakano and Asahi, 1971a and b). Mitochondrial biogenesis has also been demonstrated in the cotyledons of *Vicia faba* during germination and growth of these seeds (Dixon et al., 1980). For more detail on mitochondrial biogenesis in a variety of species, the reader is referred to reviews by Gonzalez-Cadavid (1974), Leaver (1975), Leaver and Forde (1980), Locker and Rabinowitz (1979) and Schatz and Mason (1974).

Initiation of protein synthesis in mitochondria requires formylmethionyl-tRNA (fmet-tRNA), similar to the situation in prokaryotes. This is in contrast to the cytosol of eukaryotes where initiation requires met-tRNA (Tzagoloff, 1982). The enzyme catalyzing the formation of fmet-tRNA is N^{10}-formyltetrahydrofolate:methionyl-tRNA transformylase, abbreviated, transformylase. This enzyme has been studied and at least partially purified from bacteria (Dickerman et al., 1967; Samuel and Rabinowitz, 1974), fungal mitochondria (Bianchetti et al., 1971 and 1977; Halbreich and Rabinowitz,

1971), in addition to the chloroplasts of algae (Bianchetti et al., 1971; Crosti et al., 1977; Gambini et al., 1980; Lucchini and Bianchetti, 1980) and higher plants (Bianchetti et at., 1971; Leis and Keller, 1971; Merrick and Dure, 1971). The presence of a transformylase has also been inferred from the occurrence of fmet-tRNA in bacteria (Neale et al., 1981; Seligman and Finch, 1970) and bean chloroplasts (Burkard et al., 1969). The *Euglena* transformylase has been shown to be synthesized in the cytosol (Gambini, 1978). To this author's knowledge, there is, however, only one report in the literature concerning a higher plant mitochondrial transformylase (Guillemaut et at., 1972). The role of the transformylase, in addition to other factors involved in the initiation of protein synthesis in eukaryotes, prokaryotes and organelles, is discussed in a recent review (Kozak, 1983).

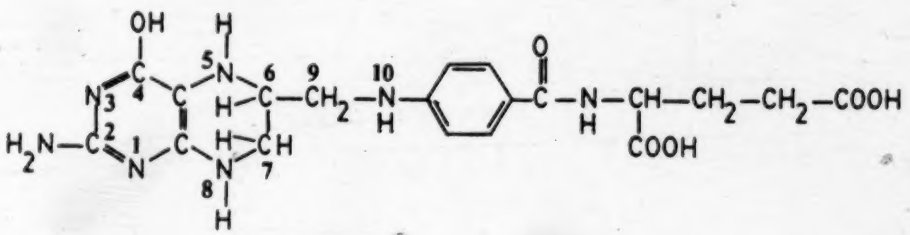
In all species examined, the source of the formyl group of fmet-tRNA has been demonstrated to be 10-HCO-H$_4$PteGlu (Staben and Rabinowitz, 1984). The final section of this Introduction is concerned with several aspects of folate biochemistry, with special reference to studies of mitochondrial folate metabolism.

The Biochemistry of Folate Derivatives

Formyltetrahydrofolate is representative of a class of compounds known as pteroylglutamates, or more commonly, as folates. These compounds are ubiquitous in living cells, and

play a key role in cellular metabolism, functioning as co-enzymes in the synthesis of purines, pyrimidines and certain amino acids (Blakely and Benkovic, 1984). Folates are also involved in methylation reactions, in addition to initiation of organelle protein synthesis (Cossins, 1980). The major pathways of folate metabolism are shown in Fig. 1. In most reactions involving folates, these compounds function as carriers of one-carbon units. A derivative of folic acid has also been identified as the acrasin of *Dictyostelium* (de Wit and Konijn, 1983).

The folate molecule, shown below, consists of a



pteridine ring, a *p*-aminobenzoic acid moiety and an L-glutamic acid residue. One-carbon (C_1) units bind to the 5 and 10 positions. Naturally-occurring derivatives are normally highly-reduced, having hydrogen at the 5, 6, 7 and 8 positions. In addition, native folates contain a chain of glutamate residues, joined by gamma-glutamyl linkage. These derivatives are known as pteroylpolyglutamates, polyglutamyl

folates or, more simply as polyglutamates. It is now generally accepted that polyglutamates are the biologically-active form of folates. The properties and functions of polyglutamates have recently been reviewed by Kisliuk (1981) and McGuire and Bertino (1981).

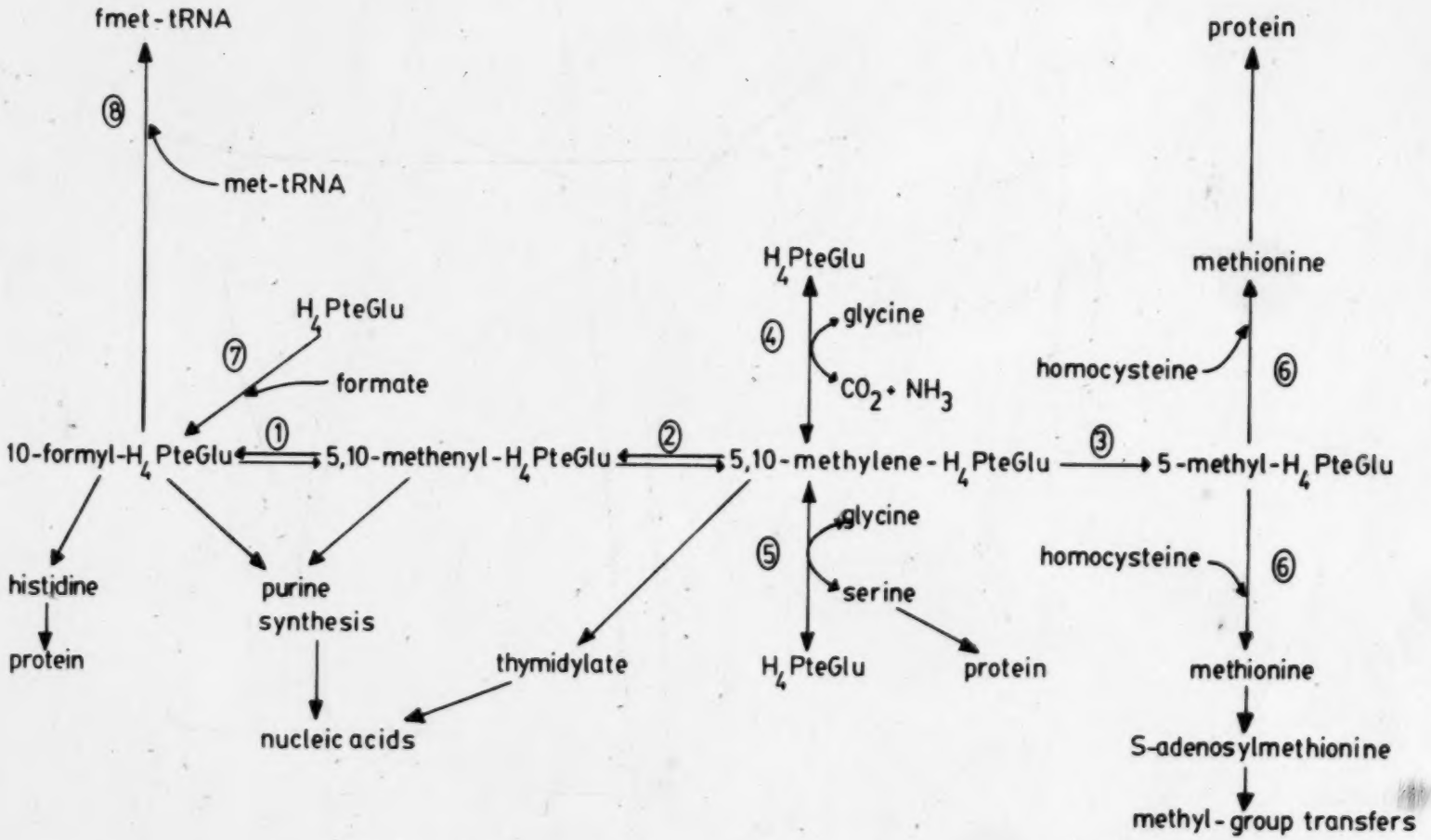
Folate derivatives at the formyl, methenyl and methylene oxidation levels are freely interconvertible. This is accomplished enzymatically and the requisite enzymes have been detected in plants (Cossins, 1980). Therefore, C_1 units can enter the folate pool at any one of these oxidation levels and be available for syntheses that are folate-dependent. Formation of CH_3-H_4PteGlu from CH_2-H_4PteGlu (Fig. 1, reaction 3) is, in general, an irreversible process, although in some species of bacteria CH_3-H_4PteGlu can enter the folate pool (Shane and Stokstad, 1977). Interconversion of folate derivatives is shown in Fig. 1 (reactions 1-3).

Studies on the occurrence and metabolic roles of folates have been performed in many organisms since the initial discovery of this compound over forty years ago. Early research was summarized in a recent article (Welch, 1983). The occurrence of folates in various tissues is discussed in greater detail by Scott and Weir (1976), Cossins (1980 and 1984), Blakely (1969) and Blakely and Benkovic (1984).

The occurrence and metabolism of folates has also been studied in plants, and this research has been recently

Figure 1. Major Pathways Of Folate Metabolism

Reaction Number	Trivial Enzyme Name	Systematic Enzyme Name	E.C. Number
1.	5,10-Methenyltetrahydrofolate cyclohydrolase	5,10-Methenyltetrahydrofolate 5-hydrolase (decyclizing)	3.5.4.9
2.	5,10-Methylenetetrahydrofolate dehydrogenase	5,10-Methylenetetrahydrofolate:$NADP^+$ oxidoreductase	1.5.1.5
3.	5,10-Methylenetetrahydrofolate reductase	5-Methyltetrahydrofolate:NAD^+ oxidoreductase	1.1.1.68
4.	Glycine decarboxylase	5,10-Methylenetetrahydrofolate:ammonia hydroxymethyltransferase	2.1.2.10
5.	Serine hydroxymethyltransferase	5,10-Methylenetetrahydrofolate:glycine hydroxymethyltransferase	2.1.2.1
6.	Methionine synthase	5-Methyltetrahydrofolate:L-homocysteine methyltransferase	2.1.1.13
7.	10-Formyltetrahydrofolate synthetase	Formate:tetrahydrofolate ligase	6.3.4.3
8.	Methionyl-tRNA transformylase	10-Formyltetrahydrofolate:L-methionyl-tRNA N-transformylase	2.1.2.9


fmet-tRNA
⑧
met-tRNA
H4PteGlu
⑦
formate
10-formyl-H4PteGlu
①
5,10-methenyl-H4PteGlu
②
5,10-methylene-H4PteGlu
③
5-methyl-H4PteGlu
H4PteGlu
④
glycine
CO2 + NH3
protein
methionine
homocysteine
⑥
histidine
protein
purine
synthesis
nucleic acids
thymidylate
⑤
glycine
serine
H4PteGlu
protein
homocysteine
⑥
methionine
S-adenosylmethionine
methyl-group transfers

reviewed (Cossins, 1980). Plants have the capacity to synthesize folates, and key enzymes involved in this synthesis in pea have been studied in some detail (Ikeda and Iwai, 1975; Iwai and Ikeda, 1975). Measurement of folate derivatives in pea cotyledons during germination and growth revealed a large increase in the levels of these compounds (Roos and Cossins, 1971). Treatment of cotyledons with folate antagonists such as aminopterin and methotrexate provided evidence for a net synthesis of folates during germination. Inhibitor treatment also strongly curtailed seedling growth.

The general requirement for folate derivatives, especially in rapidly dividing cells, implies that mechanisms exist to bring C_1 units into the folate pool to supply this demand. Generation of C_1 units has been studied in several organisms, including plants (Cossins, 1980). The major sources of C_1 units appear to be glycine, serine and formate. Figure 1 (reactions 4, 5, and 7) shows the enzymes involved in the generation of C_1 units. The 2-carbon of glycine enters the folate pool via the activity of glycine decarboxylase (Fig. 1, reaction 4). In this reaction, glycine is cleaved and one molecule each of $5,10\text{-}CH_2\text{-}H_4PteGlu$, CO_2, and NH_3 are formed. In photosynthetic tissues exhibiting photorespiration, this reaction is the principal source of CO_2 loss (Tolbert, 1980). Glycine decarboxylase is localized in mitochondria and has been shown to be present in pea cotyledon

mitochondria (Clandinin and Cossins, 1975).

Methylene-H$_4$PteGlu can also be generated from serine through the activity of serine hydroxymethyltransferase (Fig. 1, reaction 5). This reaction forms glycine in addition to the C$_1$ unit, and has been demonstrated in plants (Shah et al., 1970; Kisaki et al., 1971).

N^{10}-formyl-H$_4$PteGlu is generated directly via 10-formyltetrahydrofolate synthetase (Fig. 1, reaction 7). Evidence exists for its presence in pea and it appears to be mainly localized in the cytosol (Iwai et al., 1967; Suzuki and Iwai, 1974), although significant activity has also been detected in pea mitochondria (Clandinin and Cossins, 1972).

Enzymes of folate interconversion have been studied in plants. Methenyl-H$_4$PteGlu cyclohydrolase (Fig. 1, reaction 1) was demonstrated to be widely distributed in plants (Suzuki and Iwai, 1970). These authors have shown that methylene-H$_4$PteGlu dehydrogenase is also present in plants and on the basis of differential centrifugation studies, concluded it was localized largely in the cytosol (Suzuki and Iwai, 1974). The rather old organelle extraction technique used by these authors (Wilson and Shannon, 1963) and the lack of respiratory activity and marker enzyme data, however, suggests that the levels of particulate folate metabolism indicated are minimum values. As will be outlined in the following paragraphs, significant folate metabolism in organelles has been demonstrated.

Although most studies of folate metabolism have been performed at the whole cell level, several lines of evidence indicate mitochondria have discrete folate pools and an active folate metabolism. The levels and nature of folates in these organelles have been examined in a number of species. Studies of mitochondria isolated from the livers of rat and guinea pig showed the principal folate derivatives to be 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu (Wang et al., 1967; Corrocher and Hoffbrand, 1972, resp.), with the former predominating. McClain et al. (1975), however, demonstrated that mitochondria from Swiss mouse brain contained only 5-CH_3-H_4PteGlu. These authors have suggested that this unusual pool is due to the specialized nature of these cells. The compartmented nature of folate metabolism in mammalian cells was confirmed in more recent reports (Shin et al., 1976).

Mitochondria extracted from 4-day old pea mitochondria contained 5- and 10-HCO-H_4PteGlu, in addition to small amounts of 5-CH_3-H_4PteGlu (Clandinin and Cossins, 1972). These results are in contrast to the situation in whole cotyledons, where 5-CH_3-H_4PteGlu formed the majority of the pool (Roos and Cossins, 1971). Clandinin and Cossins (1972) also demonstrated the ability of isolated mitochondria to incorporate labelled formate, glycine and 5-CH_3- and 5,10-CH_2-H_4PteGlu into folate derivatives as well as amino acids related to C_1 metabolism. In a later paper, these authors established that pea mitochondria were able to

synthesize methionine, a process requiring 5-CH_3-H_4PteGlu (Clandinin and Cossins, 1974). Enzymes involved in folate synthesis have also been demonstrated to occur in pea mitochondria (Ikeda and Iwai, 1970, 1975; Okinaka and Iwai, 1970). Folate derivatives and enzymes involved in folate metabolism are also present in chloroplasts (Cossins, 1980).

From the data outlined above, it is clear that folate metabolism in plants is at least partially compartmented and that mitochondria have the ability to synthesize folates and to generate, interconvert and utilize one-carbon units via an active folate metabolism. The presence in pea mitochondria of methionine synthesis as well as formyl-H_4PteGlu synthetase activity indicates substrates for the transformylase can be generated endogenously and thus illustrates a connection between folate metabolism and mitochondrial protein synthesis.

The Present Study

As discussed in the preceding sections, seeds exhibit large increases in mitochondrial respiratory activity during germination and subsequent growth. The development of this activity may occur via activation of organelles present in cotyledons or may result from increases in the number of mitochondria (Bewley and Black, 1978). Previous studies have attempted to differentiate between these two possibilities by employment of selective inhibitors of cytosolic and mitochondrial protein synthesis. The present study

approached this problem from a different perspective. The activity of 10-formyltetrahydrofolate:methionyl-tRNA transformylase, a key enzyme involved in the initiation of organelle protein synthesis, was examined during germination and growth of pea and peanut seeds. This enzyme would be expected to be active only in those organelles exhibiting protein synthesis, a process essential for biogenesis. The ability of isolated pea mitochondria of different ages to synthesize proteins was also examined.

The levels and nature of mitochondrial folate derivatives were also studied over the same time period. The folate pool supplies one of the substrates of the transformylase, namely 10-HCO-H$_4$PteGlu polyglutamate. The level of this derivative may be expected to change in parallel with changes in enzyme activity. The present study, therefore, has examined the ability of pea and peanut mitochondria to synthesize proteins during germination and growth, in addition to measurement of this process in pea mitochondria.

As noted above, the two species used in the present study were pea and peanut. Pea was chosen for several reasons, the most important being the numerous literature citations for research on this species. Furthermore, earlier work from this laboratory examined several aspects of folate metabolism in germinating pea seeds (e.g. Cossins et al., 1972; Dodd and Cossins, 1968, 1969, 1970; Roos et al., 1969; Roos and Cossins, 1971) and in pea mitochondria (Clandinin

and Cossins, 1972, 1974, 1975). There are also numerous studies on the mobilization of storage reserves and respiratory activity in pea. From a practical perspective, this species is also easily grown uniformly in sufficient quantity and the cotyledons provide a good source of mitochondria. Peanut was also chosen as it represents a developing system in contrast to that of pea, since mitochondrial biogenesis has been shown to occur in this species.

II. MATERIALS AND METHODS

Chemicals

Sodium [^{14}C]-formate, L-[^{3}H-CH$_3$]-methionine L-[U-^{14}C]-methionine, L-[^{35}S]-methionine and L-[4,5-^{3}H]-leucine were purchased from Amersham Corporation. Bulk t-RNA, from *Escherichia coli* MRE 600 was supplied by Boehringer Mannheim. Fatty-acid-poor bovine serum albumin (BSA) was obtained from Calbiochem. All other analytical-grade chemicals were supplied by either Sigma Chemical Co., St. Louis, Mo., or Fisher Scientific, Edmonton.

In experiments involving radiochemicals, liquid scintillation was used to measure radioactivity. In the course of this study, three different scintillation fluors were used. Biofluor, a pre-mixed fluor, was supplied by New England Nuclear. Bray's solution (Bray, 1960) and a toluene-based fluor were prepared using scintillation grade chemicals supplied by either Sigma Chemical Co. or Fisher Scientific. The toluene fluor contained, in 1 litre of scintillation grade toluene, 4 g PPO and 0.15 g POPOP. The choice of fluor depended on the type of sample to be analyzed.

Plant Material

Seeds of pea (*Pisum sativum* L. cv Homesteader) were purchased from Robertson-Pike, Edmonton, Alberta. Seeds of

peanut (*Arachis hypogaea* L. cv Early Spanish) were obtained from Stokes Seeds Ltd., St. Catherine's, Ontario.

Prior to imbibition, all seeds were surface sterilized by soaking in 1% sodium hypochlorite for 5 minutes. The seeds were then rinsed several times with sterile distilled water and allowed to imbibe sterile water. Pea seeds were soaked for 18 h and peanut for 6 h. Damaged or nonimbibed seeds were discarded and the remainder were planted in moist sterile, horticultural grade vermiculite and allowed to germinate and grow at 25°C in darkness for the required period of time. If necessary, plants were watered with sterile distilled water. In all experiments, zero time was the start of the soaking period.

For each experiment, seedlings of uniform size were required. Therefore, in order to determine seedling size as a function of age, batches of 100 seeds were germinated and grown for various lengths of time and the radicle length of each seedling measured. The average size for each age was then determined.

In all experiments, only cotyledon tissue was used. After the plants had been grown for the appropriate period of time, they were separated from the vermiculite, rinsed in sterile water, and allowed to stand in ice-cold water. The testa was removed and the seedling excised from each seed. In order to remove any associated bacteria, the cotyledons were soaked for 1 minute in 70% ethanol, allowed to drain, and then soaked for 1 minute in 1% H_2O_2. The cotyledons were

rinsed several times with ice-cold sterile water and allowed to stand in ice-cold water prior to grinding.

Isolation of Mitochondria and Measurement of Respiratory Activity

All steps in the procedure were performed at 4 °C. The pH of all extraction media was adjusted to 7.2 at 22 °C with 2 N KOH. The extraction media were sterilized by filtration through a 0.22 µm Millipore filter. All media were stored at -25 °C until used, with the exception of the grinding medium which was prepared one day prior to use and stored at 4 °C. All glassware and centrifuge tubes were autoclaved. All centrifugation steps were performed using a Beckman JA-20 rotor unless otherwise specified.

The method used for isolation of pea mitochondria was based on that of Johnson-Flanagan and Spencer (1981). Approximately 250-300 ml of cotyledons were ground gently for 3 minutes in a mortar with 300 ml of grinding medium consisting of 0.3 M mannitol, 5 mM EDTA, 0.5% fatty acid poor bovine serum albumin (BSA), 0.05% cysteine and 50 mM TES buffer. Large particles were removed by filtration through one layer of wet sterile Miracloth and the filtrate centrifuged for 7 minutes at 700 x g to remove starch grains and other cellular debris. The pellet was discarded and the supernatant centrifuged at 21,000 x g for 5 minutes to sediment mitochondria. The supernatant was removed by aspiration and the pellets were resuspended and combined

using a wash medium consisting of 0.3 M mannitol, 0.3% BSA and 25 mM TES. The suspension was then centrifuged at 21,000 x g for 5 minutes and the supernatant discarded. The pellet was suspended in 2.0 ml of resuspension medium consisting of 0.3 M mannitol, 4 mM $MgCl_2$ and 25 mM TES. This suspension was designated the 'crude' mitochondrial suspension. The mitochondria were further purified by discontinuous sucrose density gradient centrifugation. The crude suspension was layered onto a gradient consisting of 4.0 ml of 0.6 M sucrose and 8.0 ml of 1.6 M sucrose. Both sucrose solutions contained 50 mM TES and 0.1% BSA. Centrifugation was at 32,000 x g for 1 h in a Beckman SW40Ti rotor. The band at the interface of the two sucrose layers was removed with a Pasteur pipette and diluted slowly with a buffer solution (25 mM TES, 0.1% BSA). This suspension was centrifuged 5 minutes at 21,000 x g and the pellet suspended in 2.0 ml of resuspension medium. This was designated the 'final' mitochondrial suspension.

Peanut mitochondria were isolated using a method based on that of Morohashi et. al. (1981). The differential centrifugation steps were essentially the same as for pea except that the constituents of some extraction media were different. The grinding medium contained 0.4 M mannitol, 5 mM EDTA, 0.05% cysteine, 0.5% BSA and 30 mM TES. Wash medium consisted of 0.3 M mannitol, 1 mM EDTA, 0.1% BSA and 20 mM TES. The crude pellet was further purified using the gradient technique used for pea mitochondria.

The biochemical purity of pea and peanut mitochondria was assessed using assays of marker enzymes; catalase (Clandinin and Cossins, 1972), alcohol dehydrogenase (Racker, 1955) and succinic dehydrogenase (Cooper and Beevers, 1969). These enzymes were assayed at several stages of the purification procedure including the final suspension.

Respiratory rates, ADP:O and respiratory control ratios were routinely measured polarographically using an oxygen monitor (Yellow Springs Instrument Company Model 53), with succinate as the substrate. The reaction mixture contained, in a final volume of 3.0 ml, 0.3 M mannitol, 50 mM TES, 4 mM $MgCl_2$, 5 mM potassium phosphate, 0.75 mg/ml BSA and 8 mM succinate (Solomos et al., 1972). The final pH was adjusted to 7.2 using 2 N KOH. Several cycles of ADP addition (150 nmoles) were initiated for each sample and average values for respiratory and ADP:O ratios were calculated according to Chance and Williams (1956).

Extraction of Folate Derivatives

Folate derivatives were extracted from whole cotyledons and isolated mitochondria of pea and peanut grown over a seven day period. Cotyledonary folates were extracted according to Method 2 of Roos and Cossins (1971). A known amount (ca. 2.0 g) of peeled cotyledons was sliced quickly into 1 mm sections and placed into 2% (w/v) ascorbate solution (ascorbic acid adjusted to pH 6.0 with KOH),

previously heated in a boiling water bath for 10 minutes. The slices were incubated for 10 minutes in a boiling water bath to eliminate gamma-glutamyl carboxypeptidase activity and to initiate folate extraction. The tissue was then cooled quickly in an ice-water bath and ground in an ice-cold mortar. The brei was quantitatively transferred to a large centrifuge tube using aliquots of 0.5% (w/v) ascorbate and incubated in a boiling water bath for a further 10 minutes to complete folate extraction. Denatured protein was removed by centrifugation at 15,000 x g and the supernatant retained. The pellet was washed once with 5.0 ml of 0.5% (w/v) ascorbate and recentrifuged. The combined supernatants were stored at -25 °C.

Mitochondrial folates were extracted from suspensions of gradient-purified mitochondria, sonicated at 95% of maximum output, with a Fisher ultrasonic probe. Aliquots of a suspension were mixed with an equal volume of 20% (w/v) ascorbate, pH 6.0, and incubated in a boiling water bath for 10 minutes. Denatured protein was removed by centrifugation at 13,000 x g for 10 minutes. The pellet was washed once with 10% (w/v) ascorbate, recentrifuged, and the combined supernatants stored at -25 °C.

To test for endogenous gamma-glutamyl carboxypeptidase activity, mitochondria were incubated with yeast extract (yeast extract folates are almost entirely folylpoly-glutamates) in the following manner. A sonicated suspension of mitochondria was dialyzed overnight in 0.1M K phosphate,

pH 7.2, to remove any endogenous folate. The dialysate was incubated with yeast extract (0.02%), 1% ascorbate solution, 0.1 M HEPES pH 7.6, at 25 °C for 0, 30, 60 and 90 minutes. The reaction was stopped by incubation in a boiling water bath and the samples were stored frozen until assayed.

Microbiological Assay of Folate Derivatives

All tissue extracts and fractions recovered after DEAE-cellulose chromatography were assayed for folate content using the 'aseptic plus ascorbate' technique of Bakerman (1961). *Lactobacillus casei* (ATCC 7469) and *Pediococcus acidilactici* (ATCC 8081, formerly *P. cerevisiae*), which require folate derivatives for growth, were the organisms used in this study. Growth response, measured by titration of the lactic acid produced by these bacteria, was dependent on the level of folates present and therefore standard curves relating the volume of titrant to amounts of folates could be constructed (Freed, 1966). Levels of folates in samples were determined by reference to these curves.

Standards, samples and inoculum were added aseptically to culture tubes in a sterile transfer hood. To prevent oxidation of samples during incubation at 37 °C for 72 h, 0.6% (w/v) ascorbate solution (pH 6.8 for *L. casei*; pH 7.0 for *P. acidilactici*) was added to each tube. Each sample was assayed using at least three aliquot sizes, in duplicate. After incubation, the lactic acid produced was titrated back

to the original pH of the medium using 0.1 N NaOH.

Bacterial cultures were maintained on agar slants consisting of 2.5% yeast extract, 0.5% glucose, 0.5% Na acetate and 1.5% agar. The cultures were transferred bi-weekly to fresh slants. Bacteria for inoculation were grown overnight at 37 °C in 10 ml of inoculum broth containing 1% yeast extract, 2% glucose, 2% Na acetate, 100 ul salt solution 'a' (see below, basal media stock solutions) and 100 ul salt solution 'b' (see below). The pH was adjusted to 6.8 with NaOH, at room temperature. Cells were washed free of broth and diluted ca. 100-fold with 0.9% (w/v) NaCl. Two drops per tube of this diluted culture were used as the inoculum.

Composition of the basal assay media was based on those of Freed (1966) for *L. casei* and Bakerman (1961) for *P. acidilactici* using the modifications of Spronk (1971). The components of the two media are shown in Table 1. After mixing the ingredients, the pH was adjusted to 6.8 for *L. casei* and 7.0 for *P. acidilactici*. The manganous sulfate solution was then added and the volume made up to 1 liter. Medium (5.0 ml) was added to 20x150 mm culture tubes and autoclaved 8 minutes at 121 °C.

Stock solutions of ingredients prepared according to either Freed (1966), Hansen (1965), or Spronk (1971) were made as shown below. All solutions were stored over chloroform at 4 °C.

Table 1. Composition of basal media used in microbiological assay of folates.

	L. casei	*P. acidilactici*
Stock Solutions (ml)		
1. Casein, Acid hydrolysate	100	100
2. Casein, Enzymatic hydrolysate	0	100
3. Adenine-Guanine-Uracil	50	50
4. Asparagine	60	60
5. Manganous sulfate	20	20
6. Salt solution 'b'	10	80
7. Tryptophan	40	40
8. Vitamins	100	100
9. Xanthine	100	100
10. Folic acid	0	1
11. Tween 80	0	1
Dry reagents * (g)		
Cysteine-HCl hydrate	0.5	0.76
Glucose, anhydrous	40	40
Glutathione, reduced	0.005	0.005
Sodium acetate, anhydrous	40	0
Sodium citrate	0	35
KH_2PO_4, anhydrous	1.0	4.0
K_2HPO_4, anhydrous	1.0	4.0

*Dry reagents were first dissolved in ca. 300 ml water prior to addition. The final volume was made to 1 litre with water.

Stock solution 1. Casein, Acid Hydrolysate. This solution was prepared according to Freed (1966) except that the final pH was left at 3.5. Acid-hydrolyzed casein (100 g) was added to water and the pH adjusted to 3.5 with HCl. Niacin and residual folic acid were removed by mixing the casein with activated charcoal (20 g) for 3 h. The solution was filtered and made up to 1 liter.

Stock solution 2. Casein, Enzymatic Hydrolysate. Casein (25 g) was added to water, the pH adjusted to 3.5, and 10 g charcoal added. After stirring for 3 h, the filtrate was made to 1 liter.

Stock solution 3. Adenine-Guanine-Uracil (Hansen, 1965). 0.2 g of each compound was dissolved in 12 N HCl, and the solution made to 1 liter.

Stock solution 4. Asparagine (Hansen, 1965). L-asparagine monohydrate (5 g) was dissolved in water to make 500 ml.

Stock solution 5. Manganous sulfate (Spronk,1971). 2.0 g of $MnSO_4.H_2O$ was added to water to make 200 ml.

Stock solution 6. Salt solution 'b' (Freed,1966). This solution contained in 1 liter, 20 g $MgSO_4.H_2O$, 1 g NaCl, 1 g $FeSO_4.7H_2O$, 1 g $MnSO_4.H_2O$ and 1.0 ml of 1 N HCl.

Stock solution 7. Tryptophan (Hansen, 1965). *l*-Tryptophan (5 g) was dissolved in 50 ml of 1 N HCl and made to 500 ml.

Stock solution 8. Vitamins (Freed, 1966). This solution contained in ca. 300 ml *p*-aminobenzoic acid (10 mg),

pyridoxine-HCl (40 mg), thiamine-HCl (4 mg), calcium pantothenate (8 mg), niacin (8 mg) and biotin (0.2 mg). To this was added 10 mg riboflavin dissolved in 200 ml of water containing glacial acetic acid. In addition to this, 1.9 g Na acetate and 1.6 ml glacial acetic acid were dissolved in water. The three solutions were combined and made to 1 liter.

Stock solution 9. Xanthine (Hansen, 1965). Xanthine (0.2 g) was heated in 10 ml of conc. NH_4OH until dissolved and diluted to 1 liter.

Stock solution 10. Folic Acid. This solution (2 ng/ml) was prepared by dilution of the folic acid (PteGlu) standard stock solution (see below).

Stock solution 11. Tween 80 (Hansen, 1965). 25 g of Tween 80 (polyoxyethylene sorbitan monooleate) was dissolved in 250 ml of 95% ethanol.

Stock solution 12. Salt solution 'a'. This solution contained in a final volume of 500 ml, 25 g K_2HPO_4 and 25 g KH_2PO_4.

Standard curves were routinely prepared with each assay using solutions freshly prepared from standardized stock solutions. Folic acid (PteGlu) was used as the standard for *L. casei*, *d,l*-5-HCO-H_4PteGlu for *P. acidilactici*. The standard solutions were prepared by dissolving carefully weighed folate compounds in dilute KOH. The concentration of each derivative was determined by colorimetric assay (Shiaffino, 1959) using *p*-aminobenzoic acid as the standard.

Representative curves are shown in Fig. 2. *P. acidilactici* does not respond to *d*-5-HCO-H$_4$PteGlu, therefore growth response is given in terms of *l*-5-CHO-H$_4$PteGlu equivalents (May et al., 1951). For samples assayed with *L. casei*, results are in equivalents of folic acid (PteGlu).

Enzymatic Hydrolysis of Folylpolyglutamates

The bacterial strains commonly used in folate microbiological assay do not give a significant response to folate derivatives having greater than three glutamyl residues (Blakley, 1969). Therefore, in order to determine the levels of these derivatives, samples were subjected to hydrolysis by gamma-glutamyl carboxypeptidase, which converts folylpolyglutamates to mainly folylmonoglutamates. Some di- and triglutamyl folates were also formed (Blakley, 1969).

For this study, the carboxypeptidase was prepared from 3-day old pea seedlings. Peeled cotyledons (10 g) were ground in 20 ml of ice-cold 0.1 M K phosphate buffer, pH 6.0. The brei was filtered through Miracloth and centrifuged for 7 minutes at 3,000 x g to remove cellular debris. The supernatant was dialzyed overnight in the same buffer, at 4 °C. The dialyzed extract was stored, for a maximum of 6 months, at -25 °C. The extract was diluted 10-fold with 0.1 M K phosphate, pH 6.0 before use. Significant folate levels could not be detected in the enzyme solution when measured by microbiological assay.

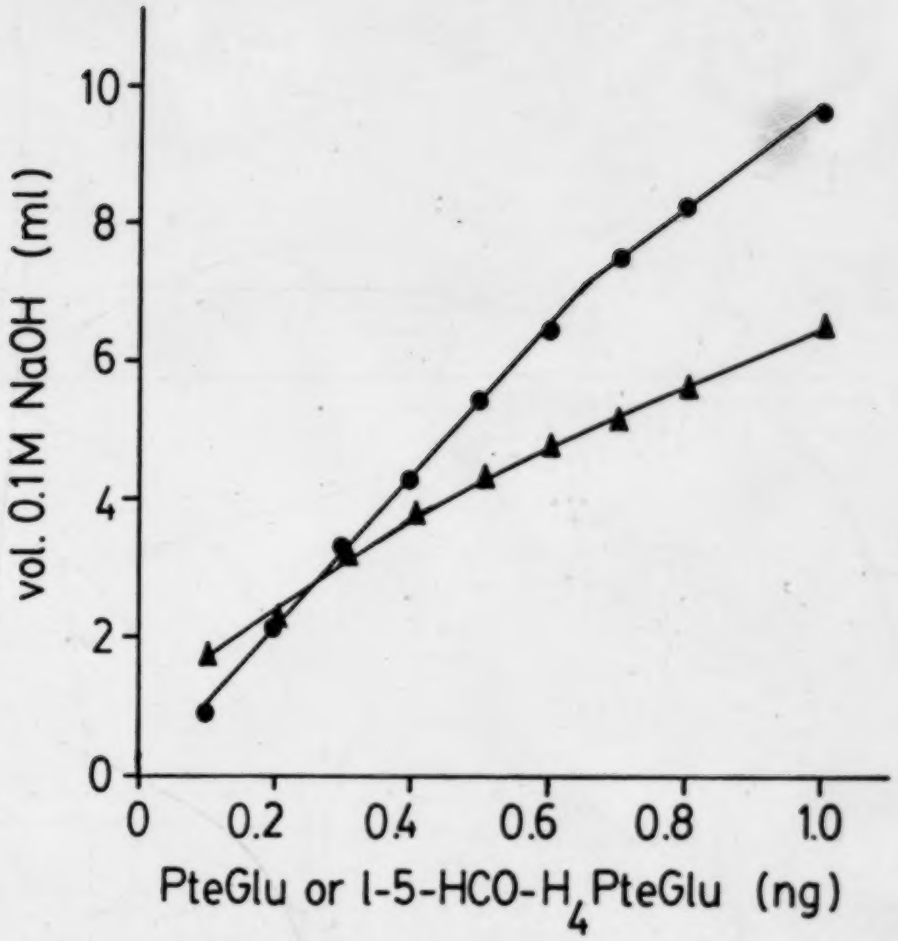


Figure 2. **STANDARD CURVES OF GROWTH RESPONSE OF L.CASEI TO PTEGLU AND P.ACIDILACTICI TO L-5-HCO-H,PTEGLU**
Growth response was measured by titration of lactic acid produced during a 72 h incubation at 37 °C. Each point represents the mean of two determinations. Circles, *L. casei* response, triangles, *P. acidilactici* response.

Hydrolysis of folylpolyglutamates was performed by incubating a folate sample in 0.1 M Na acetate, pH 4.5 containing 5% ascorbate and gamma-glutamyl carboxypeptidase, for 5 h at 37 °C. A sample of yeast extract was routinely taken through the same procedure to verify carboxypeptidase activity. A minimum 10-fold increase in assayable folates after enzyme treatment indicated an active enzyme. The reaction was stopped by incubation at 95 °C for 2 minutes.

DEAE-Cellulose Chromatography of Mitochondrial Folates

Mixtures of folate derivatives were separated using DEAE-cellulose chromatography. Columns (1.8x20 cm) were prepared according to Sotobayashi (1966). Samples of carboxypeptidase-treated mitochondrial folates were added to the column and washed into the column bed with two 3 ml washes of 0.6% (w/v) ascorbate solution, pH 6.0. The derivatives were separated by elution of the column with a gradient from 0.6% (w/v) ascorbate, pH 6.0 to 0.5 M K phosphate plus 0.6% (w/v) ascorbate, pH 6.0 (Sotobayashi, 1966). Fractions (3.0 ml) were collected in tubes containing 100 µl of 10% (w/v) ascorbate, pH 6.0 (to prevent oxidation of folates), and were stored frozen. Identification of the various derivatives was by reference to previous work that used an identical separation system, in addition to chromatography of authentic standards of 5-HCO- and 10-[^{14}C]-HCO-H$_4$PteGlu (Roos and Cossins, 1971; Clandinin and Cossins, 1972).

Assay of N'°-formyltetrahydrofolate:methionyl-tRNA transformylase

The assay system used was based on that of Crosti et al. (1977). The reaction mixture contained, in a final volume of 100 µl, 150 mM HEPES (pH 7.6), 20 mM KCl, 20 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.55 µM ^{3}H-met-tRNA (dissolved in 60 mM HEPES, pH 7.6; 15 mM KCl; 15 mM $MgCl_2$), 10 µM 10-[^{14}C]-HCO-H$_4$PteGlu and 0.2-1 mg/ml protein. The reaction was stopped after 30 minutes at 30 °C by addition of water-saturated phenol. Sodium acetate (pH 4.8) was added to yield a final Na acetate concentration of 0.3 M. The formylated product was recovered by three extractions with phenol and two ethanol precipitations (in a solid CO_2,ethanol bath), dissolved in water and assessed for radioactivity by liquid scintillation. A sonicated aliquot of a mitochondrial suspension was used as the protein source. Background radioactivity was measured using a complete reaction system minus enzyme.

The [^{14}C]-formyl-[^{3}H]-methionyl-tRNA formed using the above system, as well as [^{3}H]-met-tRNA were subjected to polyacrylamide gel electrophoresis with authentic *E. coli* bulk tRNA as a standard. The 1 mm, 12% gel prepared in 50 mM Na acetate buffer pH 4.0 was run at 11 volts/cm, until the dye front was ca. 1.0 cm from the bottom. The gel was soaked in ethidium bromide to stain the tRNA and a photograph taken under ultraviolet light to visualize the tRNA. In order to observe the radioactively-labelled tRNAs by fluorography,

the gel was treated according to Laskey (1980). In this procedure, the gel was impregnated with PPO and dried onto filter paper, and exposed to Kodak XAR-5 film. The film was developed after 72 h at -90 °C.

Preparation of Labelled Substrates

The two radioactively-labelled substrates used in the transformylase assay, [^{14}C]-formyl-H$_4$PteGlu and [^{3}H]-met-tRNA, were not available commercially and were therefore prepared in the laboratory. The synthesis of N^{10}-[^{14}C]-formyl-H$_4$PteGlu was based on the method of Buttlaire (1980) using a 10-formyltetrahydrofolate synthetase isolated and partially purified from *Clostridium cylindrosporum* (ATCC 7469), grown on the medium of Rabinowitz (1963).

The purification procedure used was identical to Buttlaire (1980) except that in Step 5, Sephadex G-200 was used instead of Bio-Gel A-0.5m and in Step 8, DEAE-Sephacel was used instead of DEAE-cellulose. Due to the instability of the enzyme, N^{10}-HCO-H$_4$PteGlu was prepared soon after enzyme purification using the procedure of Buttlaire (1980). The lyophilized product was dissolved in 0.1 M HCl containing 0.1 M 2-mercaptoethanol and stored at -25 °C. Acidification converts 10-HCO-H$_4$PteGlu to 5,10-CH=H$_4$PteGlu, a more stable compound (Blakley, 1969). This latter derivative was neutralized immediately prior to use with 1.0 M KOH. The concentration of 10-HCO-H$_4$PteGlu was determined

spectrophotometrically using an extinction coefficient of 24,900 $M^{-1}cm^{-1}$ at 350 nm (Buttlaire, 1980). Specific activities (dpm/umole) were calculated from a combination of radioactivity measurements and spectrophotometric determinations of concentration.

Methionyl-tRNA synthetase, isolated and partially purified from *E. coli* K12 (ATCC 10798), was used to synthesize ^{3}H-met-tRNA (Lemoine et al., 1968). Cells were grown to the end of the exponential growth phase in 10-liter quantities of Trypticase soy broth (Becton, Dickinson and Co.) using a New Brunswick Microferm fermenter. The cells were harvested using a Sharples continuous flow centrifuge. The enzyme purification procedure was modified from Lemoine et al. (1968). The initial steps were identical up to and including the Sephadex G-200 step. Two consecutive DEAE-Sephacel steps replaced the hydroxylapatite and DEAE-Sephadex procedures. As in Lemoine et al. (1968), the final product was stored at -25 °C in 50% glycerol.

Synthesis of [^{3}H]-met-tRNA was based on Lemoine et al. (1968). Conditions for optimal tRNA charging were studied and included buffer type, time course and the effects of tRNA concentration, protein and the ratio of Mg^{2+} to ATP using ^{14}C-methionine and a standard filter paper assay. In the present study, HEPES replaced Tris-HCl and 2-mercapto-ethanol replaced reduced glutathione. The charging system consisted of 100 mM HEPES (pH 7.6), 10 mM $MgCl_2$, 10 mM KCl, 2 mM 2-mercaptoethanol, 2 mM ATP, 60 A_{260} units/ml *E. coli*

bulk tRNA, 22 μM L-[^{3}H-methyl]-methionine (specific activity 510 μCi/μmole) and ca. 240 μg/ml enzyme. After 20 minutes at 37 °C, [^{3}H]-met-tRNA was separated from the reaction system using three extractions with phenol and an overnight ethanol precipitation at -25 °C. The ^{3}Hmet-tRNA was routinely prepared one day prior to a transformylase assay.

The concentration of [^{3}H]-met-tRNA was determined from the specific activity of the [^{3}H]-methionine used. Unlabelled methionine was added to [^{3}H]-methionine (specific activity 93 Ci/mmole) to yield a final specific activity of 510 μCi/μmole. The [^{3}H]-methionine was periodically examined using a Beckman Model 121 Automatic Amino Acid Analyzer to determine its radiochemical purity.

Difference Spectra of Isolated Mitochondria

Spectral analysis of cytochromes present in isolated mitochondria was performed using the method of Collins and Bertrand (1978). Gradient-purified mitochondria were pelleted at 15,600 x g in an Eppendorf microcentrifuge (Model 5414) and resuspended in 1.1 ml of 50 mM Tris-Cl, pH 7.7 containing 5 mM EDTA. The suspension was divided into two equal portions and 1 volume of 5% deoxycholate was added to each, to lyse the mitochondria. Organelle debris was removed by centrifugation and the supernatants retained. The cytochromes in one cuvette were oxidized by potassium ferricyanide and reduced in the other by sodium dithionite. Difference spectra (reduced-oxidized) were recorded in a Pye

Unicam Model SP8-100 Spectrophotometer at room temperature. A base line was determined by scanning prior to addition of ferricyanide or dithionite.

Amino Acid Incorporation by Isolated Mitochondria

The incorporation of L-[^{3}H]-leucine into cold TCA-precipitable material was based on the method of Forde et al. (1979). The reaction mixture contained, in a final volume of 500 μl, 0.25 M mannitol, 12.5 mM HEPES (pH 7.2), 15 mM $MgCl_2$, 12.5 mM KCl, 20 mM malate (pH 7.2), 2 mM ADP (pH 7.2), 25 μM amino acid mixture (minus leucine; pH 7.0) and 1 μCi [^{3}H]-leucine (136 Ci/mmole). After addition of mitochondria (ca. 750 μg protein) to initiate the reaction, samples were incubated for 45 minutes at 25 °C. After incubation, samples were centrifuged at 15,600 x g (Eppendorf microcentrifuge) for 5 minutes to sediment the mitochondria. The pellet was solubilized in 500 μl of 0.5 M NaOH for 20 minutes at 37°C and samples were stored overnight at 4 °C after addition of 200 μl of 50% (w/v) TCA. Precipitates were pelleted by centrifugation, washed twice with 5% (w/v) TCA and hydrolyzed in 500 μl of 0.5 M NaOH for 60 minutes at 45 °C. Samples of the hydrolysate were assessed for radioactivity by liquid scintillation in Biofluor. A time course and protein curve were routinely run with each assay. Initially, to test integrity of mitochondria during assay, the respiratory activity of a sample of mitochondria of each age was determined after

isolation as well as after incubation at 25 °C for 45 minutes. Since only a small decrease was customarily observed, respiratory activity was subsequently measured after isolation only, to ensure that the activity of the mitochondrial suspension was within the normal range for that age.

Bacterial protein synthesis has many properties in common with organelle protein synthesis and therefore the presence of bacteria may have affected the results of amino acid incorporation studies. Numbers of bacteria were estimated by plating serial dilutions of a sample onto Plate Count Agar (Difco), in triplicate. The plates were incubated for 48 h at 37 °C and colonies counted. A sample of mitochondrial suspension-plus-reaction system was incubated with each assay of amino acid incorporation.

Measurement of Radioactivity by Liquid Scintillation

All radioactive samples were counted in a Tracor Analytic Liquid Scintillation counting system (Mark III, Model 6881) A separate quench correction curve, prepared using radioactive standards, was constructed for each isotope used in order to calculate disintegrations per minute (dpm). Biofluor was used for transformylase and labelled amino acid incorporation assays. The toluene fluor was used in the filter paper assays during optimization of the met-tRNA charging reaction. Bray's solution (1960) was used to survey column fractions where data on only relative

amounts of the compounds separated were required.

Protein Determination

In most cases, protein was determined using the method of Lowry et al. (1951). Protein samples containing high levels of 2-mercaptoethanol were assayed using the method of Bradford (1976). Bovine serum albumin (Calbiochem, Fraction V) was used as the standard in both assays. Standard curves were prepared for each assay.

III. Results

Organelle Isolation

A majority of the research presented in this thesis is concerned with metabolic events occurring in pea and peanut mitochondria. Therefore, it was necessary to determine the biochemical purity of mitochondria obtained using the isolation techniques described in the previous section. Marker enzymes were assayed at several stages in the isolation of pea (Table 2) and peanut (Table 3) mitochondria. Alcohol dehydrogenase was used as a marker for the cytosol, catalase for peroxisomes and succinic dehydrogenase for mitochondria.

The specific activity of succinic dehydrogenase of both species increased steadily during the isolation procedure. The largest increase occurred after discontinuous sucrose gradient centrifugation, emphasizing the value of this step. The isolation procedure used, sacrifices the quantity of mitochondria in order to obtain higher quality organelles. To attain this, portions of the high-speed pellets were discarded, in addition to the supernatants. This practice removed some mitochondria, as well as contaminating substances, and was at least partly responsible for the low yields of mitochondria; 11.3% and 12% of total units, for pea and peanut, respectively.

The bulk of the units of catalase and alcohol dehydrogenase activity appeared in the high-speed

Table 2. Distribution of marker enzymes during isolation of pea mitochondria

Fractionation Step	Alcohol dehydrogenase			Catalase			Succinic dehydrogenase		
	Sp.* act.	Total Units ($X10^{-2}$)	%**	Sp. act.	Total Units ($X10^{-6}$)	%	Sp. act.	Total Units	%
1. Low speed supernatant	12.4	346	100	98.3	27.4	100	0.12	665	100
2. High speed supernatant	14.7	459	133	6670	20.9	76.2	0.08	312	46.9
3. Crude pellet in wash medium	35.1	66.6	19.3	3080	5.8	21.4	0.31	131	19.7
4. Gradient-purified mitochondria	6.5	15.4	0.44	652	1.5	5.6	1.95	75	11.3

* Sp. act. = specific activity expressed in units/mg protein. 1 unit = µmoles product formed per hour.

** = percent of total units.

The extraction procedure was as described in Materials and Methods except that cysteine was omitted from grinding medium when succinic dehydrogenase was assayed. Three-day old cotyledons were used as as source of mitochondria.

Table 3. Distribution of marker enzymes during isolation of peanut mitochondria

Fractionation Step	Alcohol dehydrogenase			Catalase			Succinic dehydrogenase		
	Sp.* act.	Total units ($X10^{-2}$)	%**	Sp. act.	Total units ($X10^{-3}$)	%**	Sp. act.	Total units	%
1. Low speed supernatant	2.43	100	100	699	2555	100	0.12	515	100
2. High speed supernatant	3.02	116	116	1276	3841	150	0.03	128	24.8
3. Crude pellet in wash medium	1.30	0.56	0.56	5480	234	9.2	2.80	120	23.3
4. Gradient-purified mitochondria	0.83	0.09	0.09	13800	121	4.8	5.6	62.5	12

* Sp. act. = specific activity expressed in units per mg protein. 1 unit = µmole product formed per hour.

** - percent of total units.

Assay procedures as described in Table 2.

supernatant obtained from pea and peanut cotyledons (Tables 2 and 3). This would be expected from an isolation procedure designed to select for mitochondria and against other components. It was concluded that the small amount of alcohol dehydrogenase activity remaining after gradient centrifugation, less than 0.5% for pea and peanut, was insignificant.

Although the specific activity of catalase was higher after gradient centrifugation than in the low speed supernatant (pea and peanut), in pea, higher specific activities were observed in the high-speed supernatant (Table 2). In addition, the specific activity of succinic dehydrogenase increased more than 2.5 times compared to the increase in catalase specific activity. In peanut, (Table 3) the specific activity of catalase after gradient centrifugation represented an approximately 19-fold increase over the low speed supernatant values. Again, similar to the situation in pea, the increase in succinic dehydrogenase specific activity was 2.5 times that of catalase. These results indicate that although there was some peroxisomal contamination of the mitochondria, it was minimal. Electron microscopy of 3-day, gradient-purified mitochondria, supported this contention, as few peroxisomes could be detected. Furthermore, the density of peroxisomes would allow them to be pelleted by the gradient centrifugation procedure used in the present study (Quail, 1979). As a result, the catalase detected may have adhered to the

mitochondria during isolation.

Respiratory Activity of Isolated Mitochondria

It is generally accepted that respiratory activity of isolated mitochondria, in particular ADP/O and respiratory control (RC) ratios, can be used to determine mitochondrial integrity (Tzagoloff, 1982). Respiratory rates in addition to ADP/O and RC ratios were therefore determined for mitochondria isolated from 3-day-old plants. The results of these measurements are included in Figures 3 and 4. Once it was established that active mitochondria could be routinely isolated, respiratory parameters were examined in mitochondria from seeds grown over a 7-day period (Figures 3 and 4). In peanut, ADP/O ratios increased almost 2-fold during the first 3-4 days and respiratory rates rose by a slightly greater amount (Figure 4). The same trend was observed in pea, although the values are somewhat lower (Figure 3). The increases noted above for ADP/O and RC ratios were paralleled by changes in the respiratory rate of mitochondria isolated from both species.

Cytochrome oxidase, the final protein in the electron transport chain, transfers electrons moving along the chain to oxygen, forming water. The catalytic components of cytochrome oxidase are cytochromes a and a_3. These two cytochromes can be detected spectrophotometrically. Difference spectra were determined for 1- and 3-day pea mitochondria (Figure 5). The levels of cytochromes b and

FIGURE 3

CHANGES IN THE RESPIRATORY ACTIVITY OF MITOCHONDRIA ISOLATED FROM PEA COTYLEDONS OVER A 7-DAY PERIOD

Respiratory rate (upper graph) and respiratory control ratios (O——O) and ADP/O ratios (●——●) (lower graph) were determined polarographically using succinate as substrate. Respiratory rate was measured during state 3 respiration. The values were determined from 3 replicates per experiment with at least 2 separate extractions of mitochondria. The points represent mean values $\pm$ S.E.M.

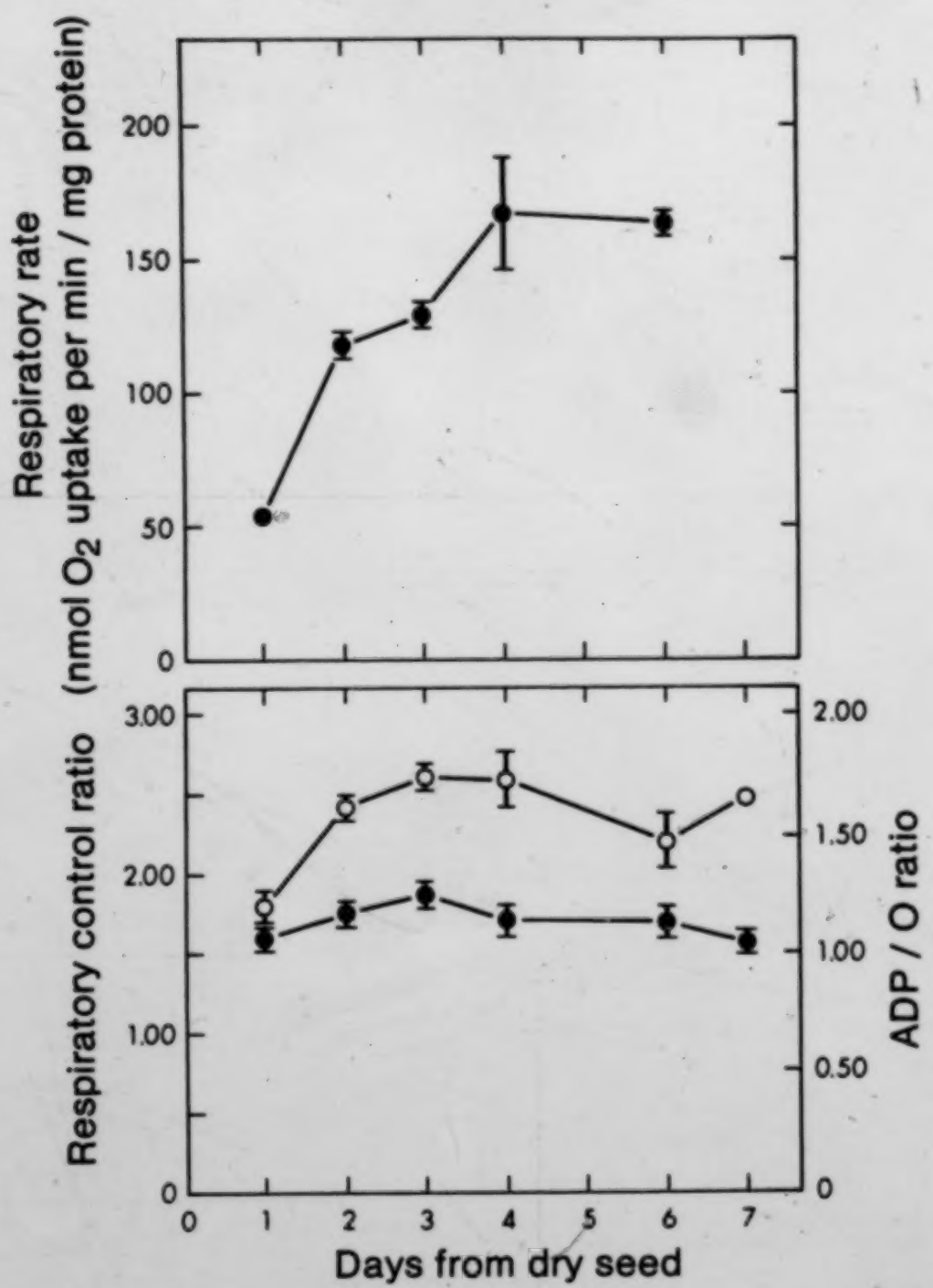

Respiratory rate
(nmol O_2 uptake per min / mg protein)
200
150
100
50
0
Respiratory control ratio
3.00
2.00
1.00
0
ADP / O ratio
2.00
1.50
1.00
0.50
0
0 1 2 3 4 5 6 7
Days from dry seed

FIGURE 4

CHANGES IN THE RESPIRATORY ACTIVITY OF ISOLATED PEANUT MITOCHONDRIA OVER A 7-DAY PERIOD

Values were determined as for pea mitochondria. Lower graph: respiratory control ratio (O——O), ADP/O ratio (●——●). Clearly reproducible, coupled mitochondria were not observed prior to the second day.

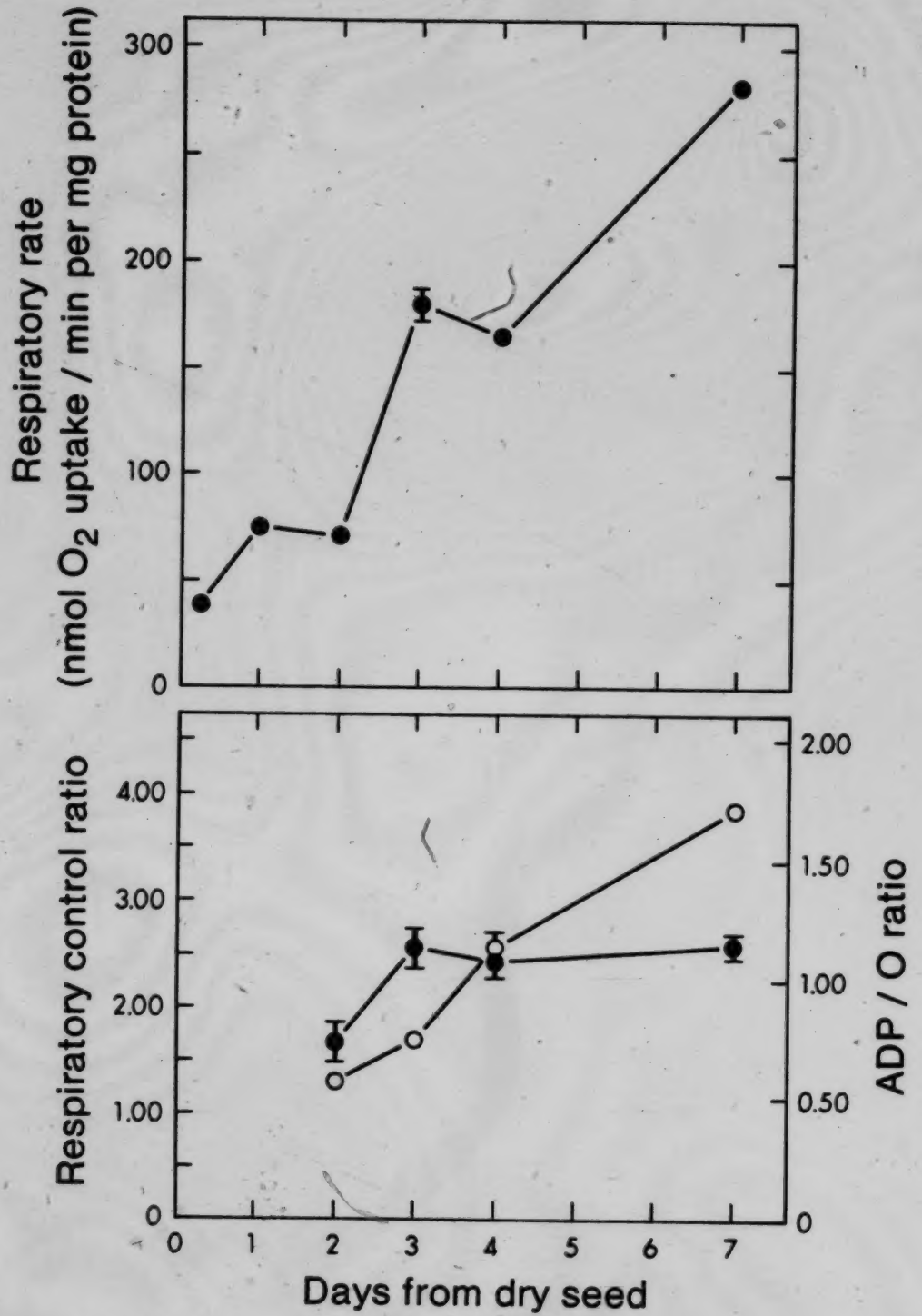
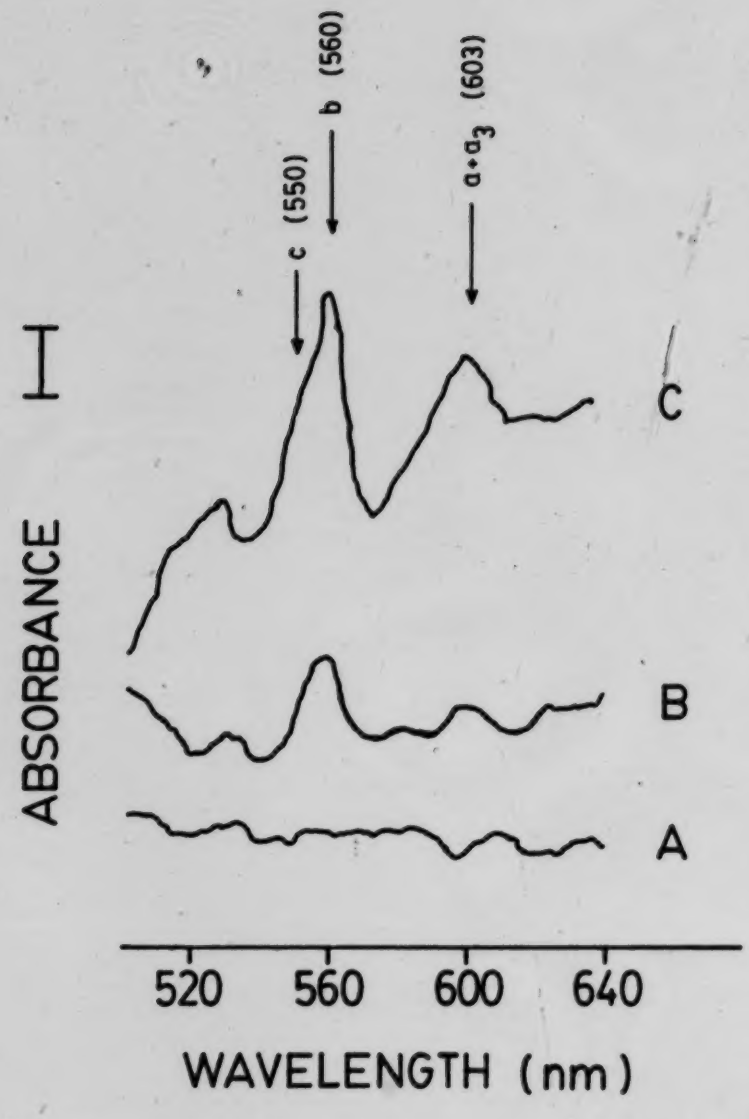

Respiratory rate
(nmol O_2 uptake / min per mg protein)
300
200
100
0
Respiratory control ratio
4.00
3.00
2.00
1.00
0
ADP / O ratio
2.00
1.50
1.00
0.50
0
0 1 2 3 4 5 6 7
Days from dry seed

FIGURE 5

AGE-RELATED CHANGES IN DIFFERENCE SPECTRA OF MITOCHONDRIA ISOLATED FROM PEA COTYLEDONS

Mitochondria were isolated and treated as described in Materials and Methods. Dithionite-reduced, ferricyanide-oxidized difference spectra were scanned as described. A, base-line obtained prior to addition of dithionite or ferricyanide; B, difference spectrum from 1-day mitochondria; 28.5 mg protein; C, difference spectrum from 3-day mitochondria; 19.0 mg protein. The bar represents 0.01 absorbance units. The spectra are direct tracings from actual scans. The arrows indicate the position of the alpha bands of the cytochromes shown and the numbers in parentheses are the wavelenghths of the absorption maxima.


c (550)
b (560)
a+a3 (603)
C
B
A
ABSORBANCE
520
560
600
640
WAVELENGTH (nm)

$a+a_3$ increased during the first 3 days as shown by the higher absorption at the relevant wavelengths, in 3-day mitochondria. The difference between 1- and 3-days is probably even more pronounced since the 1-day scan contains 1.5 times the protein used in the 3-day scan. The wavelengths for the absorption maxima of the cytochromes (alpha bands) were taken from Ikuma (1972) and Palmer (1976).

Levels and Nature of Folates in Pea and Peanut

Folate levels in both species were measured in extracts of whole cotyledons (Figures 6 and 7) and mitochondria (Figures 8 and 9), over a 7-day period. As noted in the Materials and Methods, the extracts were assessed for folates by microbiological assay using *L. casei* and *P. acidilactici*, before and after gamma-glutamyl carboxypeptidase (conjugase) treatment. *L. casei* responds maximally to all naturally-occurring folate derivatives with no more than three glutamyl residues (Blakley, 1969). Therefore assay by *L. casei*, of a conjugase-treated sample, yields a value for total folates. Samples assayed prior to conjugase treatment yield values for free folates. *P. acidilactici*, however, shows significant response to only highly-reduced, non-methylated folates having a glutamyl chain length of one or two. This differential response was used to elucidate the nature of the folate pools.

FIGURE 6

CHANGES IN PEA COTYLEDON FOLATES DURING THE FIRST SEVEN DAYS OF GROWTH

Folate levels were determined by microbiological assay using *L. casei* (open symbols) and *P. acidilactici* (closed circles). Samples were assayed using *L. casei* before (Δ——Δ) and after (O——O) hydrolysis with pea cotyledon gamma-glutamyl carboxypeptidase. Only hydrolyzed samples were assayed by *P. acidilactici*. Values are given as equivalents of folic acid for *L. casei*-assayed samples and *l*-N^5-HCO-H_4PteGlu for *P. acidilactici*-assayed samples. The level of folates in each sample was determined by using 3 different aliquots, in duplicate, from one extraction. A total of 3 extractions were made. Each point represents the means ± S.E.M.

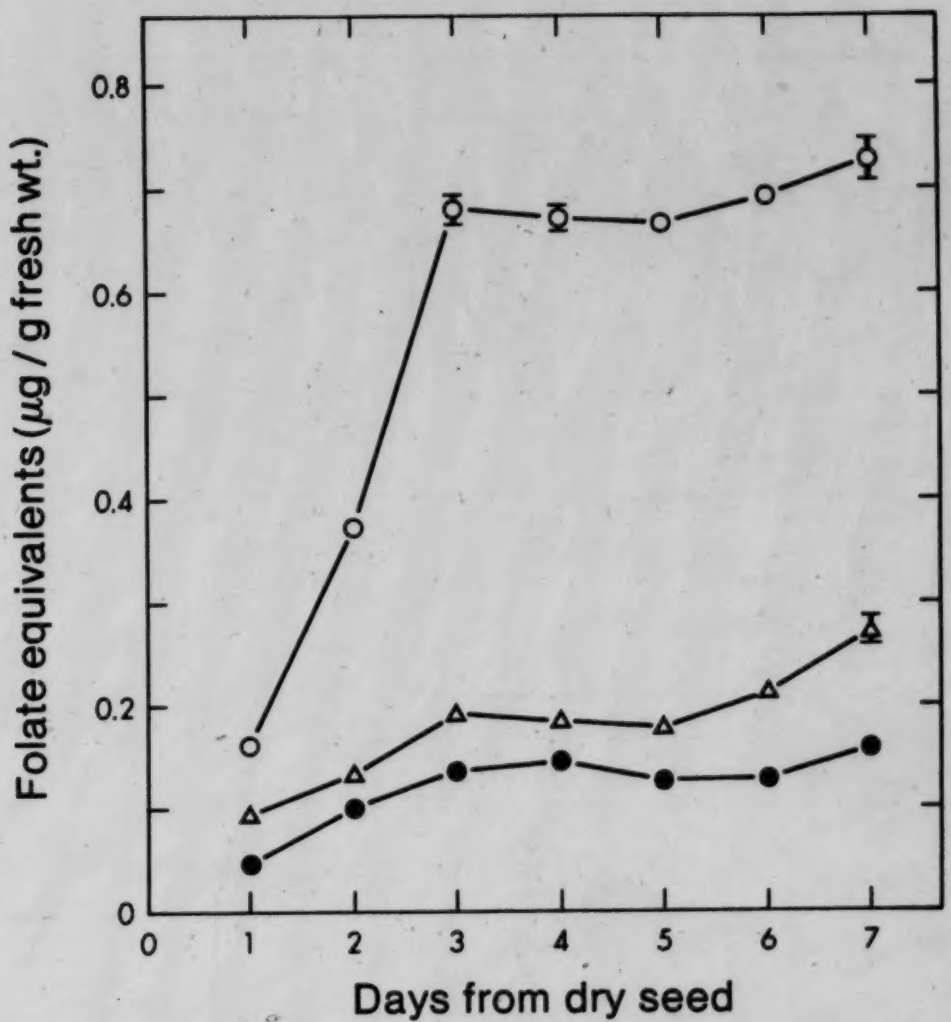
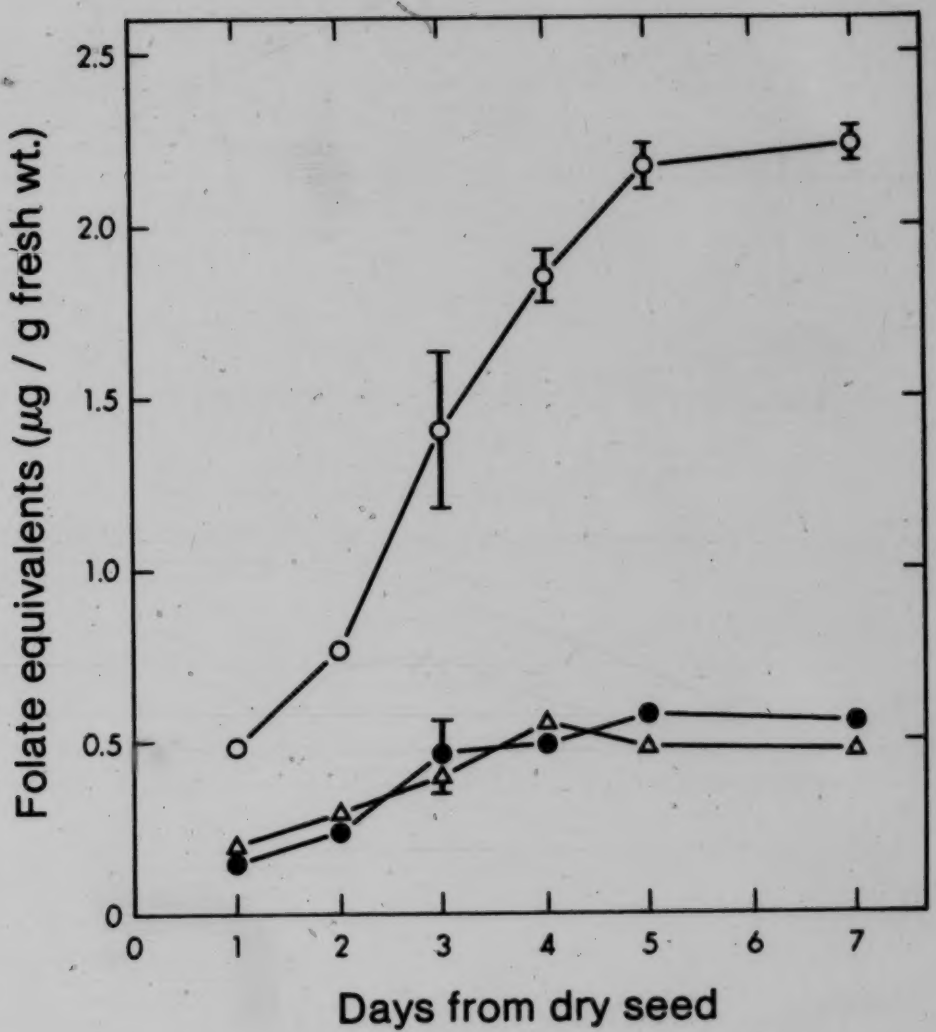

Folate equivalents (μg / g fresh wt.)
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
Days from dry seed

FIGURE 7

CHANGES IN PEANUT COTYLEDON FOLATE LEVELS DURING THE FIRST 7 DAYS OF GROWTH

The levels were determined as for pea cotyledons, with the same number of replicates. Each point represents the mean ± S.E.M. Symbols; Δ——Δ, *L. casei* before and O——O, after hydrolysis; ●——● *P. acidilactici* after hydrolysis.


Folate equivalents (µg / g fresh wt.)
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
4
5
6
7
Days from dry seed

As noted in the methods section, folate levels assayed by *L. casei* are expressed as equivalents of folic acid (PteGlu), and levels assayed by *P. acidilactici* are expressed as equivalents of *l*-5-HCO-H$_4$PteGlu. From the representative standard curves shown in Figure 2, it is clear that the response of the two species was not equal and therefore levels determined by each assay organism were not mathematically equal. However, the two sets of values can be used in studies of the nature of folate pools. The *L. casei* response to a given amount of folate was higher than *P. acidilactici* and therefore levels determined by *L. casei* appear somewhat lower than those determined by *P. acidilactici*.

Total folate levels in pea cotyledons exhibited a marked (4-fold) increase from the first to third days, and then remained relatively constant (Figure 6). Comparison of levels assayed by *L. casei* before and after conjugase treatment shows that a majority of the endogenous folate pool (at least 65-75%) was present as polyglutamyl derivatives, after the first day.

It should be noted here that the folate derivatives normally found in plant tissues are 5-HCO- and 10-HCO-H$_4$PteGlu and 5-CH$_3$-H$_4$PteGlu (Cossins, 1980). Since *P. acidilactici* does not respond to methylated derivatives, it is conceivable that levels of folates measured by this organism represent levels of formyl derivatives. When folate extracts of pea cotyledons were also assayed using *P.*

acidilactici (Figure 6), it was shown that formyl derivatives accounted for only a small portion of the total folate pool, throughout the period studied.

Peanut cotyledons were also examined for folate content over the same period of time as pea (Figure 7). Total folate levels rose ca. 4.5-fold, although maximum levels were not attained until the fifth day, as in pea. The endogenous pool consisted of at least 60-80% polyglutamates. Formyl derivatives made up only a small portion of the pool.

Folate derivatives were also assayed in pea and peanut mitochondria (Figures 8 and 9). In pea, total folates rose rapidly after imbibition, particularly after the third day (Figure 8). The rate of increase slowed after 4 days but did not reach a plateau. There was a 6-fold increase in total folates by the fourth day. Assay by *P. acidilactici* revealed that formyl derivatives made up the majority of the endogenous pool. The changes in these derivatives closely paralleled those of total folates for the first 4 days. Polyglutamyl folate levels changed during the 7-day period. These derivatives ranged from a low on the second day of ca. 25% to a high at 7 days of ca. 70%. Incubation of sonicated mitochondria with yeast extract (rich in polyglutamates) revealed no significant endogenous conjugase activity at the normal pH of mitochondrial isolation.

Total folates in isolated peanut mitochondria also rose rapidly during the first 4 days (Figure 9). *P. acidilactici* assay showed that the mitochondrial pool consisted largely

FIGURE 8

PEA MITOCHONDRIAL FOLATE LEVELS DURING GERMINATION AND GROWTH

Folates were extracted from isolated mitochondria of various ages. All samples were assayed with *L. casei* before (Δ——Δ) and after (O——O) hydrolysis by gamma-glutamyl carboxypeptidase. Hydrolyzed samples were also assayed with *P. acidilactici* (●——●). Each point represents the mean value of 3 different aliquots, in duplicate, from one mitochondrial extraction. Separate extractions yield results differing less than 10% from the above results.

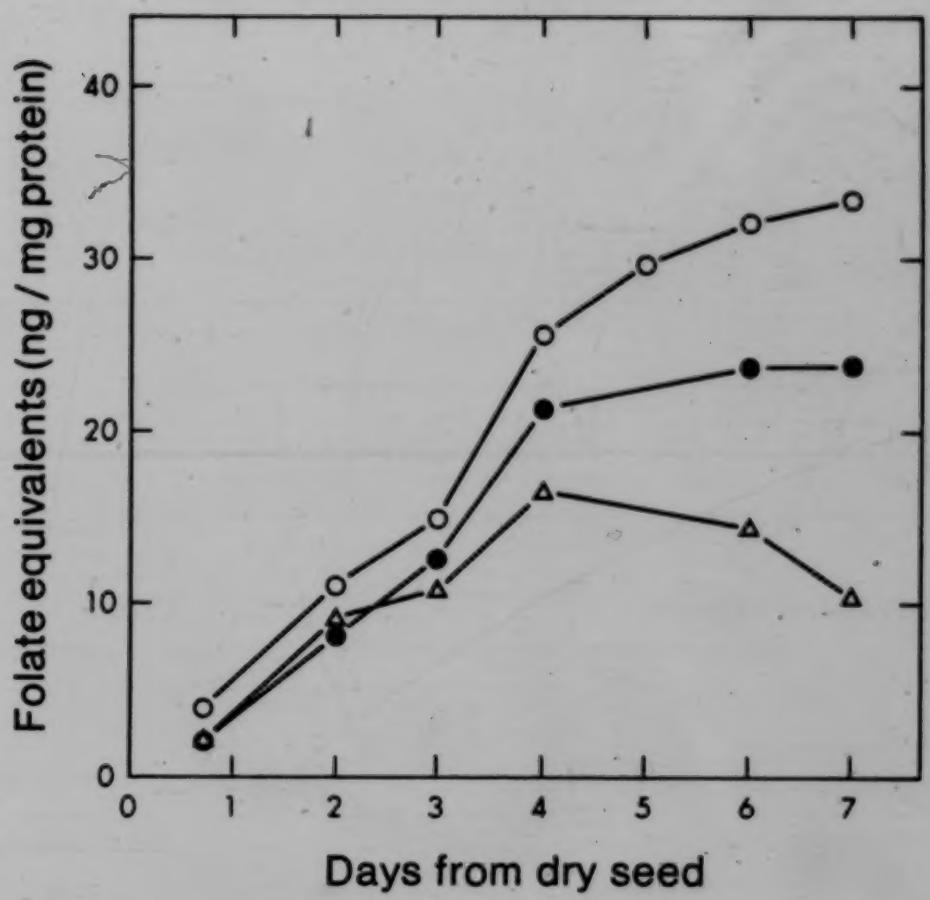
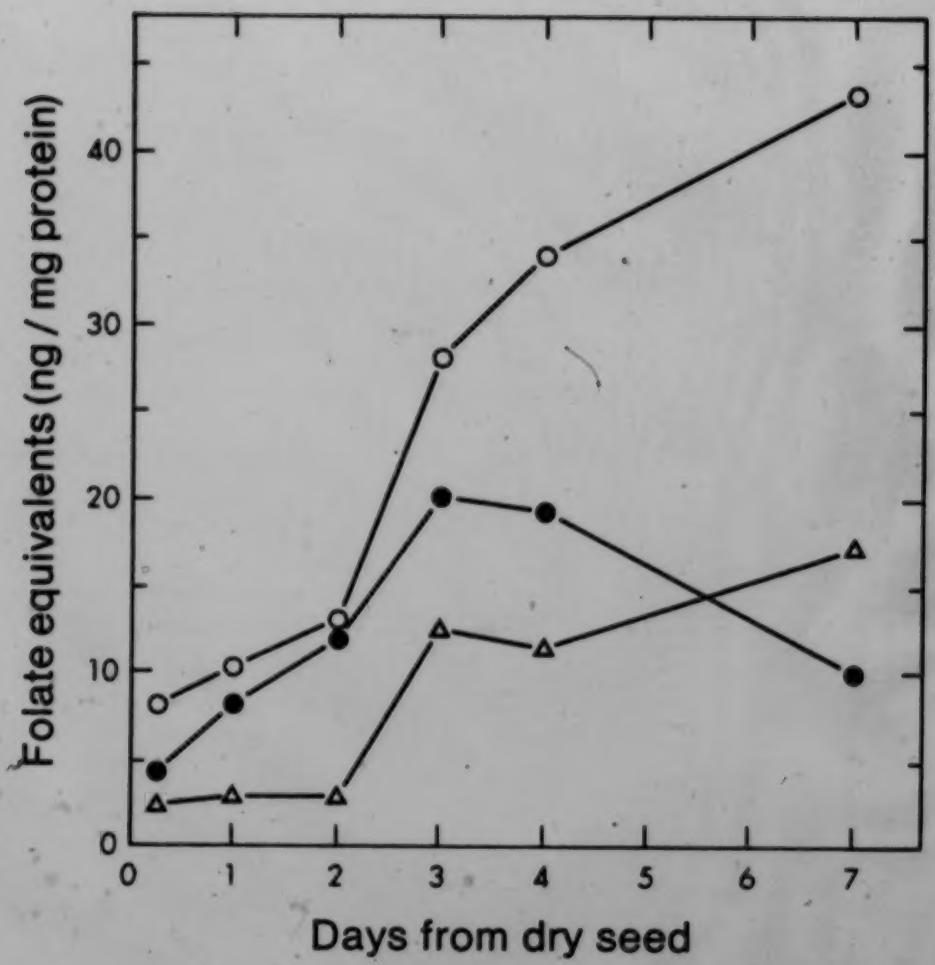

Folate equivalents (ng / mg protein)
40
30
20
10
0
0
1
2
3
4
5
6
7
Days from dry seed

FIGURE 9

PEANUT MITOCHONDRIAL FOLATE LEVELS DURING GERMINATION AND GROWTH

The levels were determined as for pea mitochondria, with the same number of replicates. Separate extractions yielded results differing less than 10% from the above results.


Folate equivalents (ng / mg protein)
0
10
20
30
40
0
1
2
3
4
5
6
7
Days from dry seed

of formyl derivatives until after the fourth day. Polyglutamyl folates made up 70-80% of the pool. No endogenous conjugase activity could be detected at the pH of mitochondrial isolation. The changes in mitochondrial total folates closely paralleled those of the cotyledons.

To examine the nature of the mitochondrial folate pools in greater detail, samples were subjected to DEAE-cellulose chromatography. Typical elution patterns of the derivatives are shown in Figures 10, 11 and 12. Identification of the monoglutamate peaks (peaks 'a', 'c', 'd') was achieved by chromatography of authentic standards of [^{14}C]-10-HCO-, 5-HCO- and 5-CH_3-H_4PteGlu. Identification of the diglutamate derivatives ('b','e','f') was by reference to previous studies from this laboratory since the monoglutamate elution patterns of the present data were identical to the earlier work (Clandinin and Cossins, 1972; Roos and Cossins, 1971).

A chromatographic separation of folates from 4-day pea mitochondria is shown in Figure 10. Aliquots of each fraction were assayed by *L. casei* and *P. acidilactici*. Assay by the former organism revealed 2 major peaks, 'a' and 'c+d'. It is known that 5-HCO- and 5-CH_3-H_4PteGlu are poorly resolved by this method (Roos and Cossins, 1971), consequently the fractions were re-assayed with *P. acidilactici*. The latter assay showed the presence of significant levels of 5-HCO-H_4PteGlu (peak 'c'), in addition to 10-HCO-H_4PteGlu (peak 'a'). Peak 'c' occurred earlier than 'c+d', and was also much sharper, indicating peak 'c+d'

FIGURE 10

DEAE-CELLULOSE CHROMATOGRAPHY OF 4-DAY PEA MITOCHONDRIAL FOLATES

Separation was as described in Materials and Methods, using a conjugase-treated sample. The fractions were assessed for folate content by microbiological assay using *L. casei* (●——●) and *P. acidilactici* (O——O). Data are expressed as equivalents of PteGlu for *L. casei* and 1-5-HCO-H$_4$PteGlu for *P. acidilactici*. No derivatives were detected prior to fraction 30.

Peak a, 10-HCO-H$_4$PteGlu

Peak b, 10-HCO-H$_4$PteGlu$_2$

Peak c, 5-HCO-H$_4$PteGlu

Peak d, 5-CH$_3$-H$_4$PteGlu

Peak e,f 5-HCO-H$_4$PteGlu$_2$ + 5-CH$_3$-H$_4$PteGlu$_2$

Peak e, 5-HCO-H$_4$PteGlu$_2$

These peaks were identified by reference to previous work using identical separation techniques, in addition to chromatography of authentic 5-HCO-H$_4$PteGlu and 10-[^{14}C]HCO-H$_4$PteGlu.

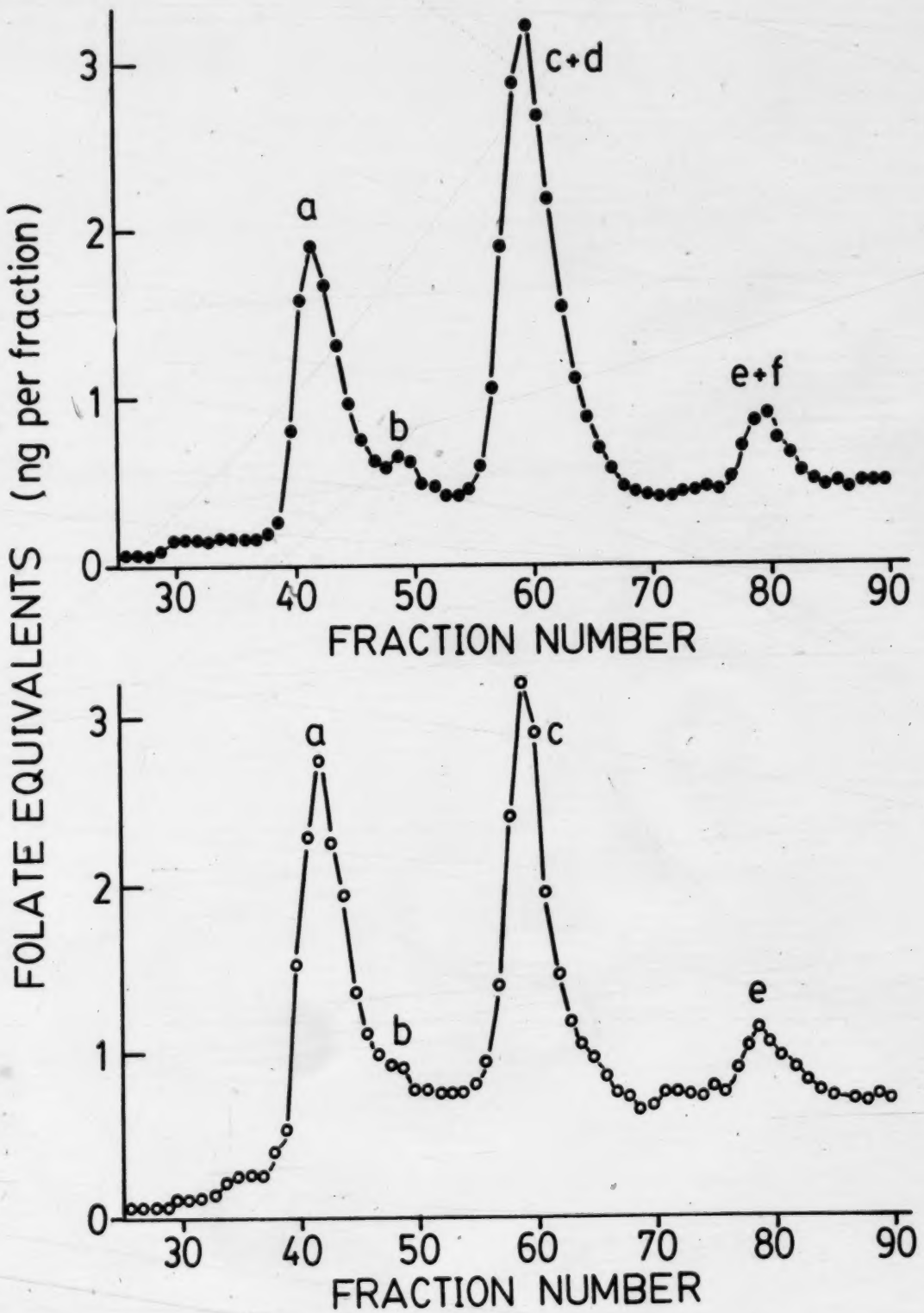

FOLATE EQUIVALENTS (ng per fraction)
3
2
1
0
a
b
c+d
e+f
30
40
50
60
70
80
90
FRACTION NUMBER
a
b
c
e
FRACTION NUMBER

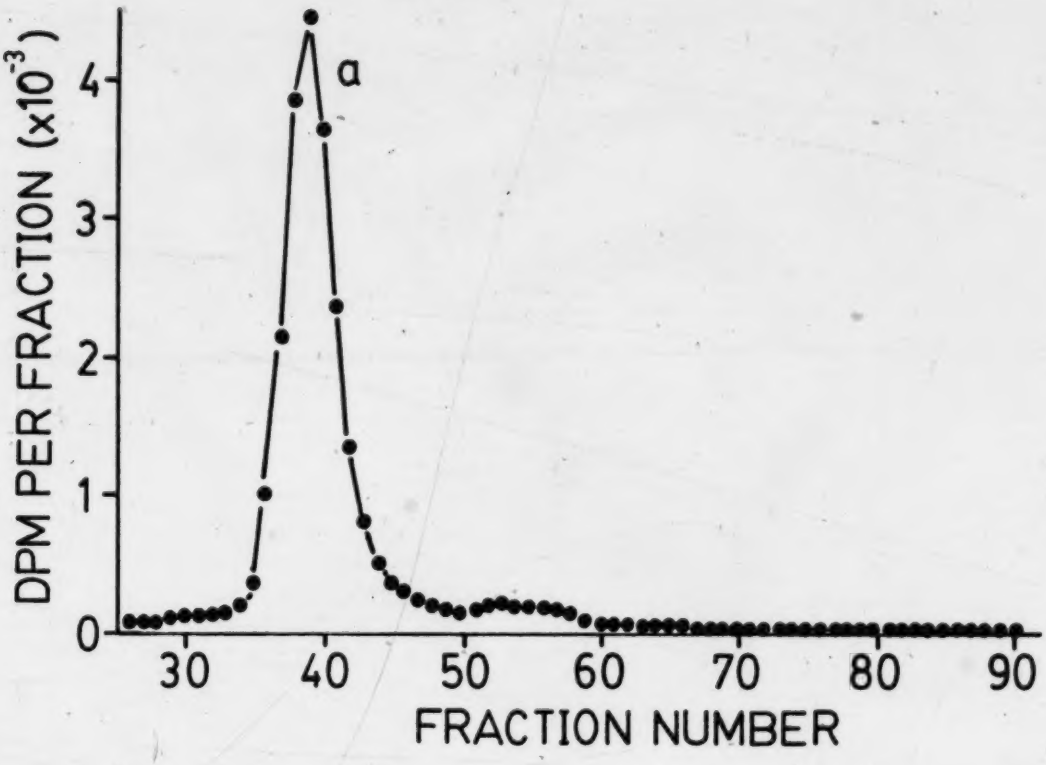
FIGURE 11

EFFECT OF HEATING ON THE STABILITY OF 10-FORMYLTETRAHYDRO-FOLATE

[^{14}C]-formyl-H$_4$PteGlu was added to ascorbate (10%), pH 6.0, and incubated in a boiling water bath for 10 minutes, cooled and added to a DEAE-cellulose column. Separation of derivatives was as described in Materials and Methods. An untreated sample of [^{14}C]-HCO-H$_4$PteGlu was also subjected to DEAE-cellulose chromatography. Untreated sample (●——●), heated sample (O——O).

Peak a, 10-HCO-H$_4$PteGlu

Peak b, 5-HCO-H$_4$PteGlu


DPM PER FRACTION (x10^{-3})
4
3
2
1
0
a
30
40
50
60
70
80
90
FRACTION NUMBER

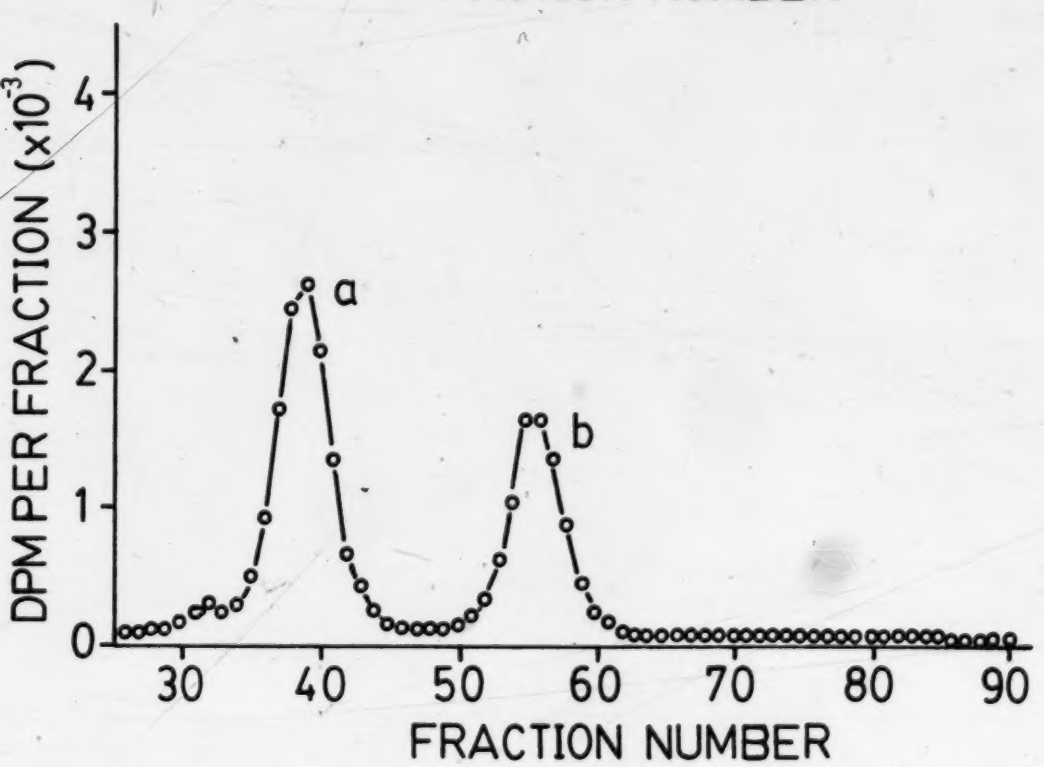

DPM PER FRACTION (x10^{-3})
4
3
2
1
0
a
b
30
40
50
60
70
80
90
FRACTION NUMBER

FIGURE 12

DEAE-CELLULOSE CHROMATOGRAPHY OF MITOCHONDRIAL FOLATES EXTRACTED FROM 4-DAY OLD PEANUT COTYLEDONS

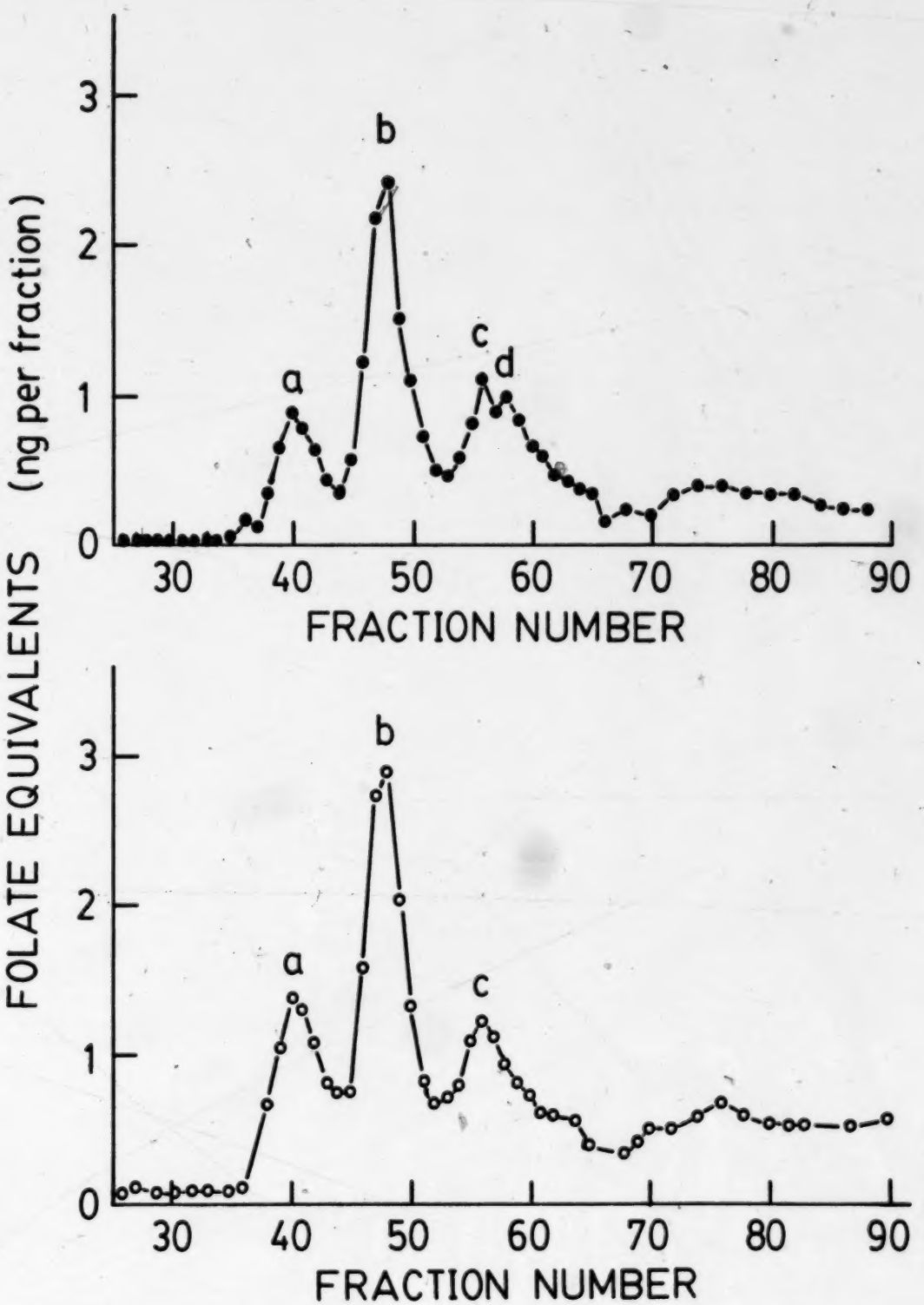
Separation was as described in Materials and Methods, using a conjugase-treated sample. The fractions were assessed for folate content by microbiological assay. Data is expressed in PteGlu (folic acid) equivalents for *L. casei* (●——●) and 1-5-HCO-H_4 equivalents for *P. acidilactici* (O——O). No derivatives were detected prior to fraction 30.

Peak a, 10-HCO-H_4PteGlu

Peak b, 10-HCO-$H_4$$PteGlu_2$

Peak c, 5-HCO-H_4PteGlu

Peak d, 5-CH_3-H_4PteGlu


FOLATE EQUIVALENTS (ng per fraction)
0
1
2
3
a
b
c
d
30
40
50
60
70
80
90
FRACTION NUMBER
FOLATE EQUIVALENTS
0
1
2
3
a
b
c
30
40
50
60
70
80
90
FRACTION NUMBER

contained 5-HCO- and 5-CH_3-H_4PteGlu. This data therefore suggests the major folate derivatives in pea mitochondria were 10-CHO-, 5-HCO- and 5-CH_3-H_4PteGlu.

The presence of 5-HCO-H_4PteGlu, in amounts almost equal to 10-HCO-H_4PteGlu, was somewhat surprising since 5-HCO-H_4PteGlu has been shown to be a competitive inhibitor of met-tRNA transformylase (Gambini et al., 1980). Conversion of 10-HCO- to 5-HCO-H_4PteGlu by high temperature, has been reported in the literature (Blakley, 1969; Wilson and Horne, 1983). Since incubation of samples in a boiling water bath is an essential step in folate extraction, the effect of this treatment on 10-HCO-H_4PteGlu stability was assessed as described in Figure 11. It is clear from this figure that heating has a significant effect, as 44% of 10-HCO-H_4PteGlu underwent isomerization to 5-HCO-H_4PteGlu. This fact must therefore be taken into consideration in any discussion of the nature of folate pools.

Calculations of ng folate/peak were made from Figure 10. After correction for background, the lower profile showed that peak 'a', (10-HCO-H_4PteGlu), represented 9.5 ng and peak 'c', (5-HCO-H_4PteGlu), 8.4 ng. Peaks 'b' and 'e' (10-HCO- and 5-HCO-H_4PteGlu$_2$, resp.) made only a small contribution to the folate pool. If it is assumed that 44% of 10-HCO-H_4PteGlu has been changed to 5-HCO-H_4PteGlu, then the value for 10-HCO-H_4PteGlu, before isomerization, would be 17 ng. Addition of the values for peaks 'a' and 'b' (lower profile) yields 17.9 ng, strongly suggesting that

almost all 5-HCO-H$_4$PteGlu present has come from 10-HCO-H$_4$PteGlu. If this assumption is valid, then the amount of 5-HCO-H$_4$PteGlu in peak 'c+d' (upper profile, Figure 10) can also be calculated. The values for ng folate/peak were found to be, peak 'a', (10-HCO-H$_4$PteGlu), 7.5 ng, peak 'c', (5-HCO-H$_4$PteGlu), 5.9 ng and peak 'd', (5-CH$_3$-H$_4$PteGlu), 8.0 ng. The 10-HCO-, 5-HCO- and 5-CH$_3$ diglutamates (peaks 'b', 'e' and 'f', resp.) made up ca. 8% of the total folate pool. It is clear from the above that the pea mitochondrial folate pool contains a majority of formyl folates.

Folate derivatives from 4-day peanut mitochondria were also subjected to DEAE-cellulose chromatography. The elution profiles from assays by *L. casei* and *P. acidilactici* are shown in Figure 12. The data shows a large percentage of the pool consisted of 10-HCO-H$_4$PteGlu$_2$. The present data also suggest that the formyl derivatives, 5-HCO-, 10-HCO- and 10-HCO-H$_4$PteGlu$_2$ made up ca. 80% of the folate pool. The *Pediococcus* data in Figure 9, however, would suggest a somewhat lower percentage. The difference in values is due, at least in part, to the response of *P. acidilactici* to 10-HCO-H$_4$PteGlu$_2$, since it is less than expected (compare the relative responses of *L. casei* and *P. acidilactici* to 10-HCO- and 10-HCO-H$_4$PteGlu$_2$). Therefore the level of folates exhibited by *P. acidilactici* is a minimal value.

10-HCO-H$_4$PteGlu:Methionyl-tRNA Transformylase Activity in Isolated Pea and Peanut Mitochondria

The two substrates for this enzyme, namely met-tRNA and 10-HCO-H$_4$PteGlu, are not commercially available as radioactively-labelled compounds and were therefore prepared enzymatically in the laboratory. The purification of met-tRNA synthetase from *E. coli* is shown in Table 4. The final purification is lower than the literature value (Lemoine et al., 1968). This is partly due to difficulties encountered in the purification procedure. The high specificity of aminoacyl-tRNA synthetases for their substrates, however, makes purification to homogeneity unnecessary (Lea and Norris, 1977). Purification of *C. cylindrosporum* formyltetrahydrofolate synthetase is shown in Table 5. The final purification is ca. 50% of the reported value (Buttlaire, 1980). The final product of both purifications, however, was more than adequate to supply the required substrates.

Once the labelled substrates were prepared, conditions for optimal transformylase assay were studied. The requirements for the pea enzyme are shown in Table 6. As expected, omission of the labelled substrates resulted in loss of activity. When Mg^{2+} was deleted, an increase in activity was observed. This was unexpected since transformylases from other sources require this cation (Crosti et al., 1977; Dickerman et al., 1967; Samuel and Rabinowitz, 1974). Some Mg^{2+} (1.5 mM) was added, however,

Table 4. Purification of *E. coli* methionyl-tRNA synthetase.

Purification Step	Protein (mg/ml)	Total Activity (units)*	Specific Activity (units/mg protein)	Yield (%)	Purification (-fold)
75,000xg supernatant	6.8	56.6	0.016	100	1
Ammonium sulfate fraction 30-50%	34	16.4	0.016	29	1
Sephadex G-200 eluate	4.6	15.2	0.033	27	2.1
DEAE-Sephacel #1	0.2	6.1	0.150	11	9.4
DEAE-Sephacel #2	0.5	6.2	0.210	11	13
Glycerol (50%) solution	1.4	5.4	0.221	10	14

* 1 unit of enzyme activity is equal to the formation of 1 μmole of met-tRNA in 10 minutes at 37°C.

Table 5. Purification of *C. cylindrosporum* formyltetrahydrofolate synthetase.

Purification Step	Protein (mg/ml)	Total Activity (units)*	Specific Activity (units/mg protein)	Yield (%)	Purification (-fold)
144,000xg supernatant	12.6	589	0.95	100	1.0
Protamine sulfate supernatant	8.6	486	0.95	82	1.0
First ammonium sulfate precipitate	5.8	433	1.14	73	1.2
Second ammonium sulfate precipitate	26	333	1.71	56	1.8
Sephadex G-200 eluate	1.4	306	2.38	52	2.5
Third ammonium sulfate precipitate	2.8	119	3.04	20	3.2
First crystallization	1.3	100	5.51	17	5.8
DEAE-Sephacel eluate	0.71	94.3	6.65	16	7

1 unit is equal to the formation of 1 μmole 5,10-methenyl-H_4PteGlu/min.

Table 6. Requirements for 10-HCO-H_4PteGlu:methionyl-tRNA transformylase activity of pea mitochondria.

Reaction System	Enzyme Activity*	%
complete	790	100
minus enzyme	n.d.	0
heated enzyme**	n.d.	0
minus K^+	632	80
minus Mg^{2+}	1122	142
minus [^{3}H]-met-tRNA	n.d.	0
minus [^{14}C]-formyl-H_4folate	n.d.	0

* Enzyme activity is expressed as pmoles fmet-tRNA formed per 100 pmoles recovered tRNA per mg protein.

**Enzyme protein was incubated in a boiling water bath prior to use.

n.d., not detected, i.e. radioactivity levels were equal to scintillation counter background. The minus enzyme system was used as the 'blank' in all cases and this value was subtracted from all other values.

with the met-tRNA for retention of RNA stability. This concentration is 5-fold lower than required by the *Euglena* transformylase (Crosti et al., 1977) and 10-fold less than the enzyme from *E. coli* (Dickerman et al., 1967). The latter two studies used pure or partially pure enzymes, whereas the present study used a crude enzyme. It is quite conceivable that Mg^{2+} was already present in the transformylase extracts in sufficient quantities.

The effect of pH on pea met-tRNA transformylase was also studied (Figure 13). The pH range chosen reflected the pH range used in assays of this enzyme from a variety of sources. From Figure 13, it is clear that, in pea, there is no significant difference in activity from pH 6.8 to 7.8. The pH chosen, 7.6, was the same as Crosti et al. (1977), the method on which the present study was based.

Studies of the effect of concentration of the two principal substrates, met-tRNA and 10-HCO-H$_4$PteGlu, are shown in Figure 14. In the case of 10-HCO-H$_4$PteGlu, a clear optimum was evident at 10 μM and this concentration was subsequently used in all assays. Enzyme activity was linear for met-tRNA concentrations up to ca. 0.5 μM. Because of slight variations encountered in the concentration of the met-tRNA, prepared enzymatically one day prior to use, it was necessary to choose a [tRNA] such that enzyme activity *vs* tRNA was linear. This allowed more accurate comparisons of enzyme activities from different assays. This is the reason enzyme activity was expressed as pmoles fmet-tRNA

Figure 13

EFFECT OF pH ON METHIONYL-tRNA TRANSFORMYLASE ACTIVITY OF 3-DAY PEA MITOCHONDRIA

Enzyme activity was measured as described in Materials and Methods. Activity is expressed as pmoles fmet-tRNA formed per 100 pmoles recovered tRNA/mg protein. Each point represents the average of two determinations. The range for each average falls within the size of the symbol.

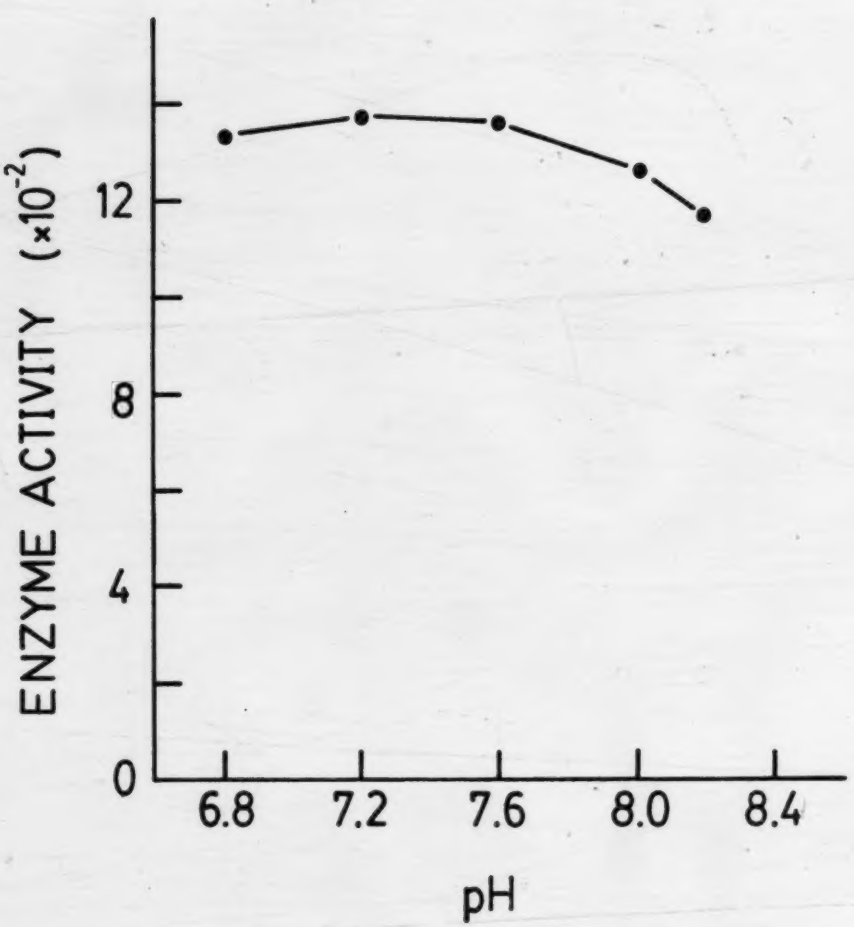
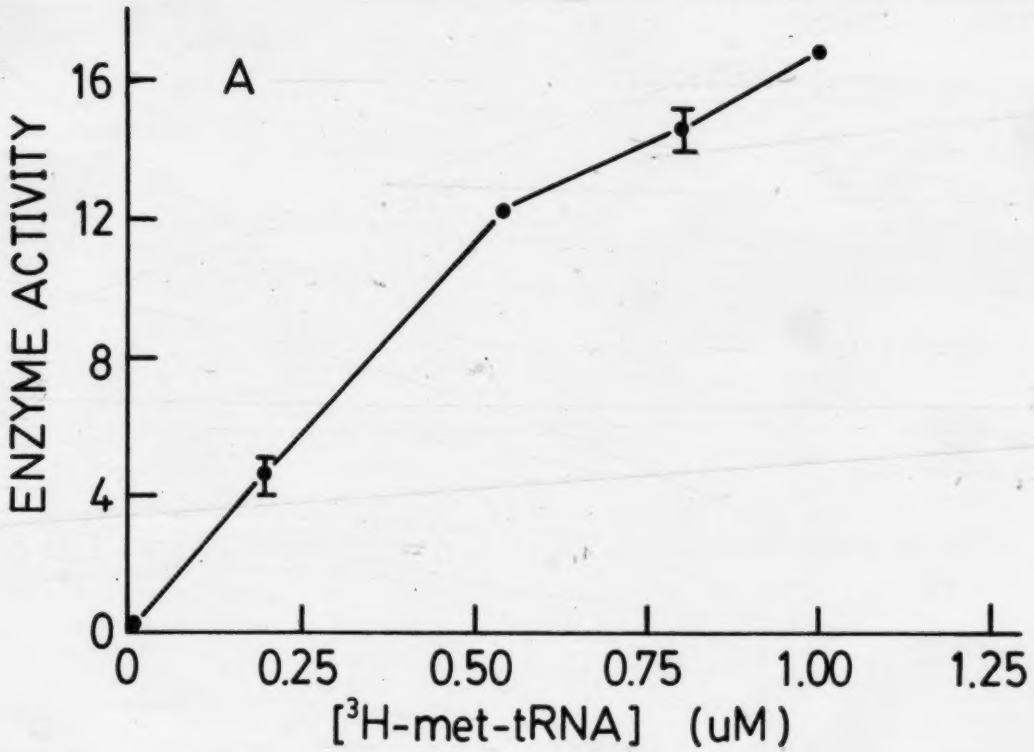
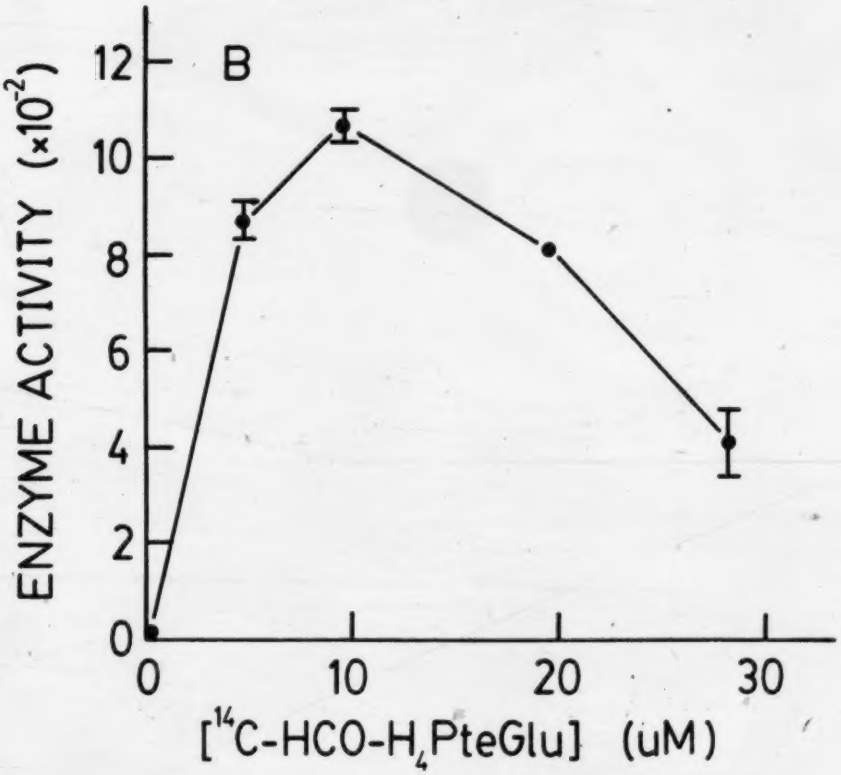

ENZYME ACTIVITY (×10⁻²)
0
4
8
12
6.8
7.2
7.6
8.0
8.4
pH

Figure 14

EFFECT OF SUBSTRATE CONCENTRATIONS ON 10-HCO-H$_4$PteGlu: METHIONYL-tRNA TRANSFORMYLASE OF PEA MITOCHONDRIA

The effect of [^{3}H]-met-tRNA (A) and [^{14}C]-HCO-H$_4$PteGlu (B) concentrations on transformylase activity were measured in 3-day mitochondria. Enzyme activity is expressed as pmoles fmet-tRNA formed in A and pmoles [^{14}C]-fmet-tRNA formed per 100 pmoles tRNA recovered/mg protein in B. Each point represents the average of two determinations. The range in values is represented by the bars.


A
ENZYME ACTIVITY
16
12
8
4
0
0
0.25
0.50
0.75
1.00
1.25
[^{3}H-met-tRNA] (uM)


B
ENZYME ACTIVITY (×10^{-2})
12
10
8
6
4
2
0
0
10
20
30
[^{14}C-HCO-H$_4$PteGlu] (uM)

formed per 100 pmoles tRNA recovered per mg protein.

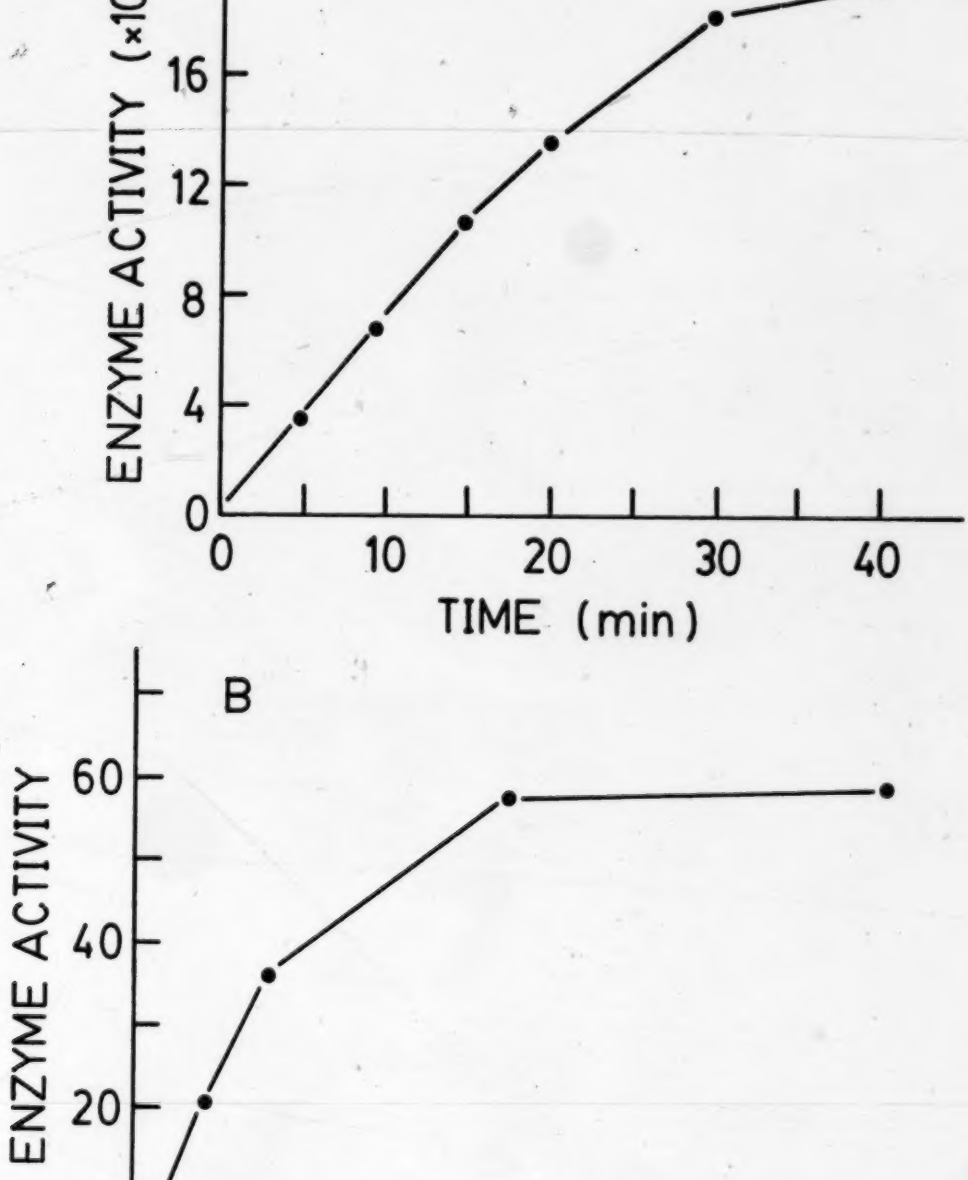
The time course of enzyme activity is shown in Figure 15. A 30 minute assay was used routinely since significant increases in enzyme activity were not observed in longer assays. Enzyme activity as a function of protein concentration is also shown in Figure 15. At least 3 concentrations of mitochondrial protein were used in each transformylase assay. This was necessary because of variations in the amount of mitochondria obtained with each extraction. It was important to assay the transformylase under conditions where activity was a linear function of protein concentration, in order to allow comparisons of enzyme activity. Only values in the linear portion of the protein curve were reported.

Conditions for optimal assay of met-tRNA transformylase were also studied in mitochondria isolated from peanut cotyledons. Requirements for activity, shown in Table 7, were very similar to the pea enzyme. The stimulatory effect of Mg^{2+} omission was also observed in peanut mitochondria. Optimal concentrations for met-tRNA and 10-HCO-H$_4$PteGlu were the same as the pea enzyme (Figure 16). The time course of enzyme activity and the effect of protein concentration are virtually identical (Figure 17) to the pea transformylase. Since the conditions for assay of the enzyme from the two sources were so similar, the effect of pH on peanut transformylase was not examined.

Figure 15

EFFECT OF PROTEIN CONCENTRATION AND TIME ON METHIONYL-tRNA TRANSFORMYLASE ACTIVITY OF 3-DAY PEA MITOCHONDRIA

Enzyme activity was measured as described in Materials and Methods. In A, activity is expressed as pmoles fmet-tRNA formed per 100 pmoles tRNA recovered/mg protein. In B, activity is expressed as pmoles fmet-tRNA formed per 100 pmoles tRNA recovered. Each point represents the average of two determinations. The range in values for each point did not exceed the symbol size.


A
ENZYME ACTIVITY (×10⁻²)
0
4
8
12
16
20
0
10
20
30
40
TIME (min)

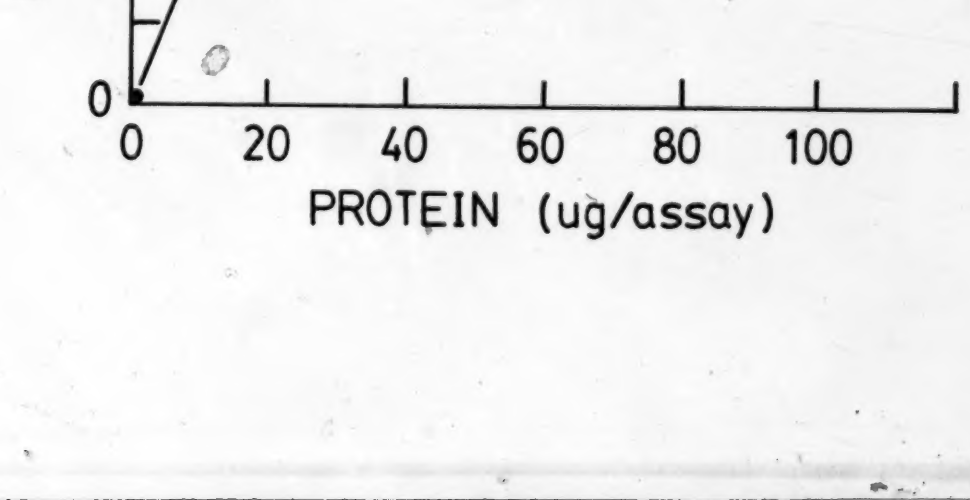

B
ENZYME ACTIVITY
0
20
40
60
0
20
40
60
80
100
PROTEIN (ug/assay)

Table 7. Requirements for 10-HCO-H_4PteGlu:methionyl-tRNA transformylase activity of 3-day peanut mitochondria.

Reaction System	Enzyme Activity*	%
complete	1535	100
minus enzyme	n.d.	0
heated enzyme	n.d.	0
minus K^+	1230	80
minus Mg^{2+}	1996	130
minus [^{3}H]-met-tRNA	n.d.	0
minus [^{14}C]-formyl-H_4folate	n.d.	0

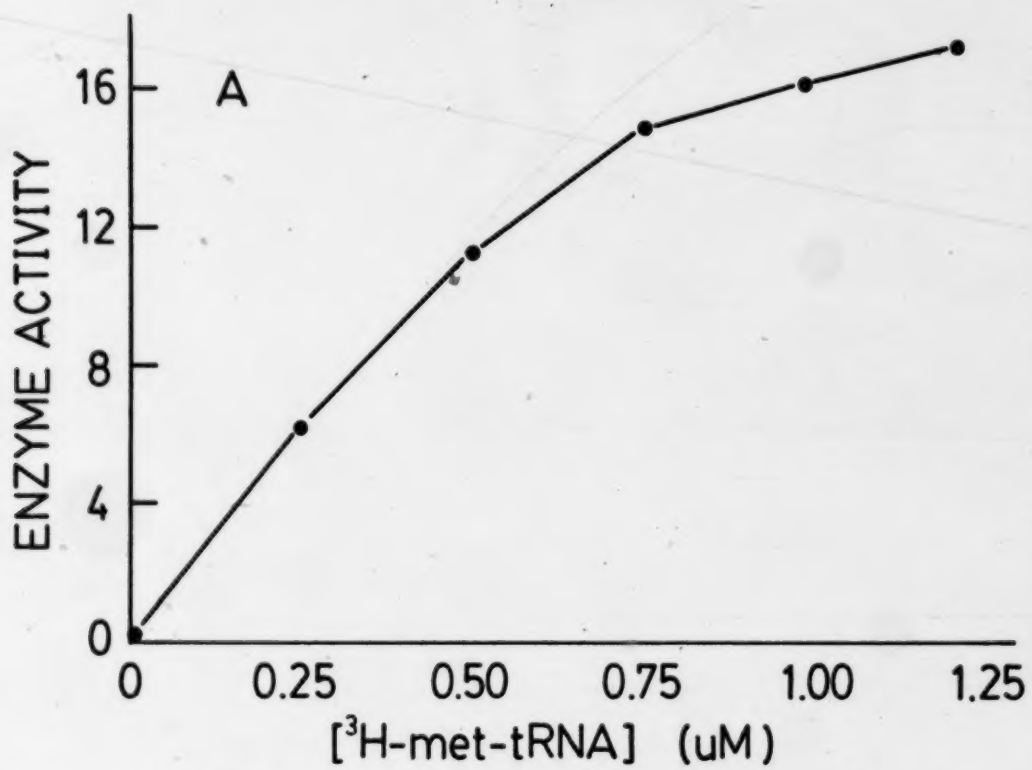
* Enzyme activity was as described in Table 6.

n.d., not detected.

Figure 16

EFFECT OF SUBSTRATE CONCENTRATIONS ON METHIONYL-tRNA TRANSFORMYLASE ACTIVITY OF 3-DAY PEANUT MITOCHONDRIA

Enzyme activity was measured as described in Materials and Methods. Activity is expressed as in Figure 14. Each point represents the average of two determinations. The bar represents the range in values. The absence of a bar at a given point indicates the range did not exceed the symbol size.


A
ENZYME ACTIVITY
16
12
8
4
0
0
0.25
0.50
0.75
1.00
1.25
[^{3}H-met-tRNA] (uM)

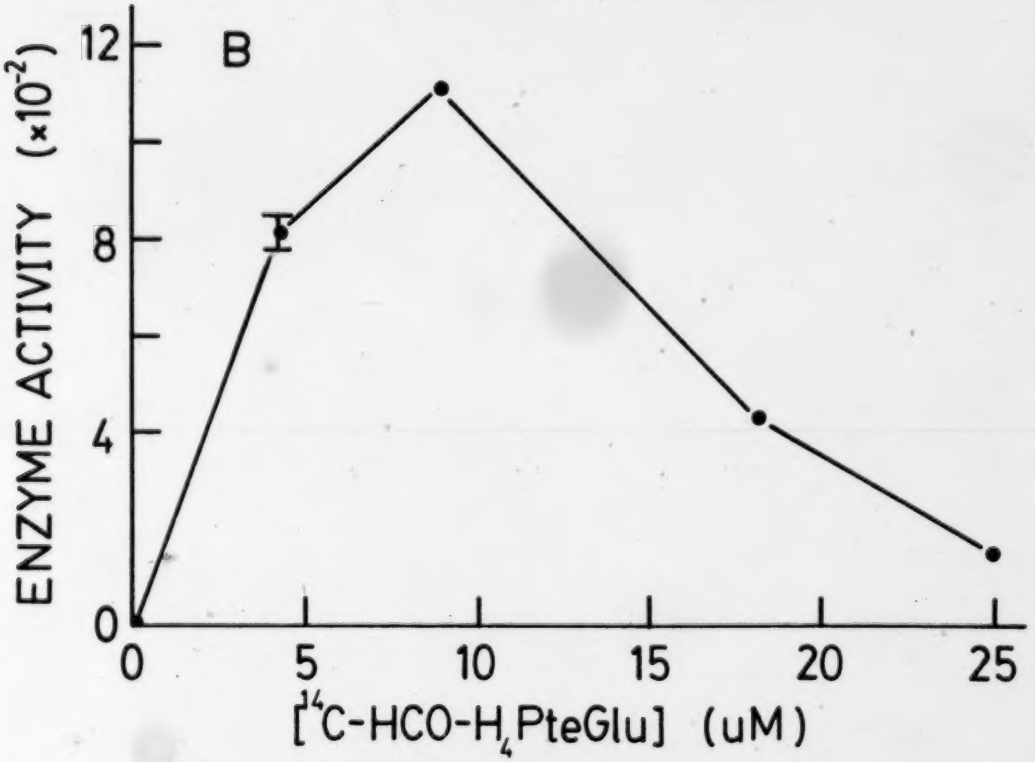
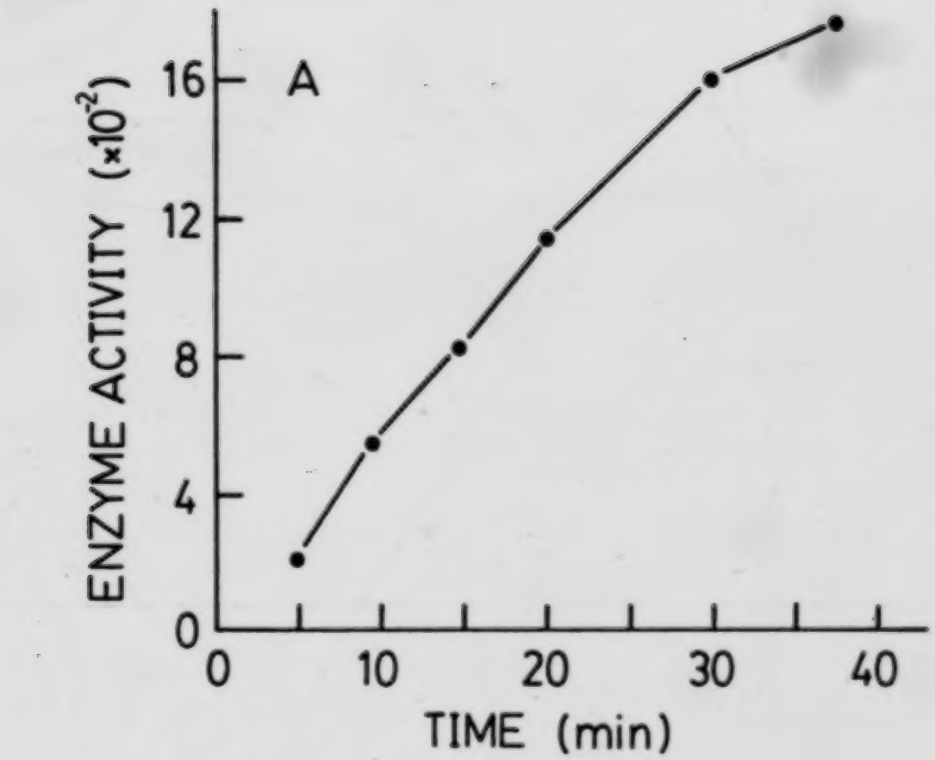
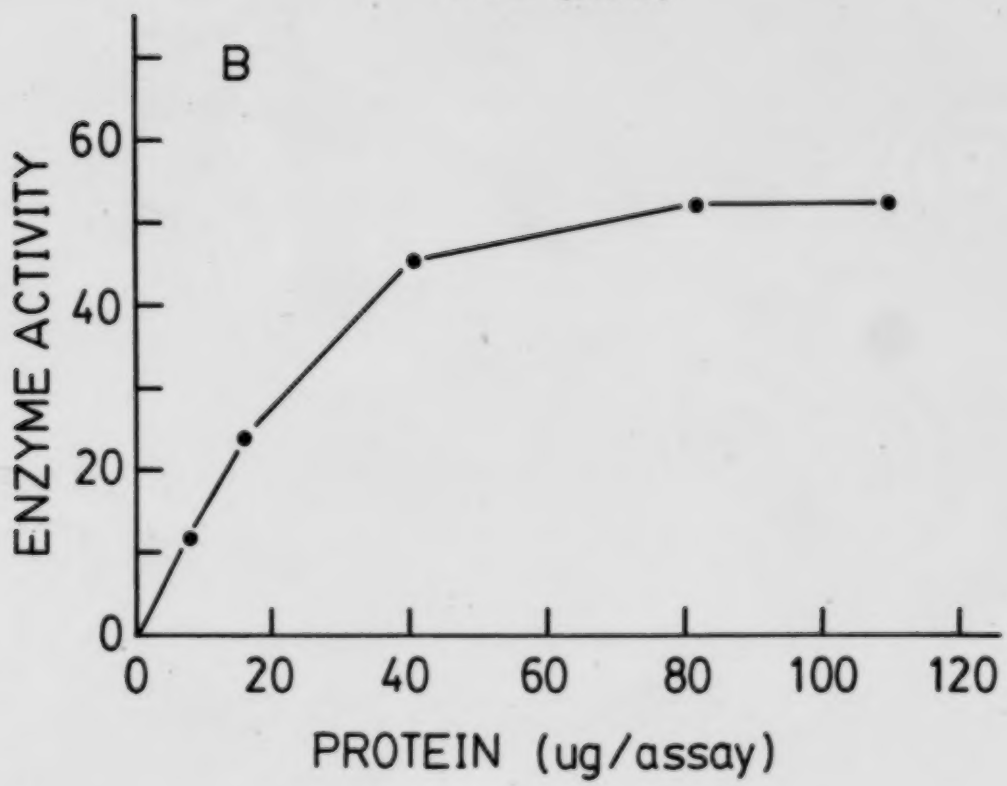

B
ENZYME ACTIVITY (×10^{-2})
12
8
4
0
0
5
10
15
20
25
[^{14}C-HCO-H$_4$PteGlu] (uM)

Figure 17

EFFECT OF PROTEIN CONCENTRATION AND TIME ON TRANSFORMYLASE ACTIVITY OF 3-DAY PEANUT MITOCHONDRIA

Enzyme activity was measured as described in Materials and Methods. Activity is expressed as in Figure 15. Each point represents the average of two determinations. The bar represents the range in values.


A
ENZYME ACTIVITY (×10⁻²)
16
12
8
4
0
0
10
20
30
40
TIME (min)


B
ENZYME ACTIVITY
60
40
20
0
0
20
40
60
80
100
120
PROTEIN (ug/assay)

Bacteria were detected in suspensions of mitochondria, particularly by the third day of growth. Since met-tRNA transformylase is also present in bacteria, it was necessary to determine if contaminating bacteria contributed to the levels of transformylase detected. The results of this study, shown in Table 8, revealed that bacteria had no significant effect on pea transformylase activity. Mitochondria isolated at earlier stages (1 and 2 days) consistently exhibited a lower level of contamination. Mitochondrial suspensions from peanut cotyledons contained similar levels of bacteria.

The product formed via the transformylase reaction was subjected to polyacrylamide gel electrophoresis. Prepared [^{3}H]-met-tRNA, and *E. coli* tRNA were also run at the same time (Figure 18). Comparison of the fluorogram, (A), with the photograph of the stained gel, (B), clearly shows that all radioactivity was associated with tRNA. The greater intensity of the fmet-tRNA band, compared to the met-tRNA band, is due to addition of the [^{14}C]-formyl group to [^{3}H]-met-tRNA.

Although the possibility still exists that the formyl group is bound to the tRNA at a location other than the methionine residue, this is unlikely. In all studies of organelle protein synthesis initiation, fmet-tRNA is required (Staben and Rabinowitz, 1984). In addition to this, fmet-tRNA has been detected in all organelles studied, including higher plant chloroplasts and mitochondria (Staben

Table 8. Possible contributions to transformylase activity by contaminant bacteria.

Protein source for transformylase assay	enzyme activity*
Bacterial suspension**	29 - 36
Mitochondrial suspension (3-day pea)	1090 - 1250

* Expressed as pmoles f-met-tRNA per 100 pmoles recovered tRNA per mg protein. The range of values from 4 replicates are shown.

**Bacteria isolated on plate counter agar from mitochondrial suspension were grown in broth as a mixed culture to near the end of the exponential phase, harvested, sonicated and treated as a mitochondrial suspension, as described in Materials and Methods. The bacterial suspension contained the number of bacteria normally detected.

FIGURE 18

POLYACRYLAMIDE GEL ELECTROPHORESIS OF FMET-tRNA

[^{14}C]-Formyl-[^{3}H]-methionyl-tRNA was prepared as in Materials and Methods. Samples of *E. coli* bulk tRNA, [^{3}H]-met-tRNA and [^{14}C]-f-[^{3}H]-met-tRNA were electrophoresed as described in Materials and Methods. A, fluorogram of the dried gel. B, photograph of the gel taken under ultraviolet light. The photographic prints were adjusted to the same size to allow direct comparison.

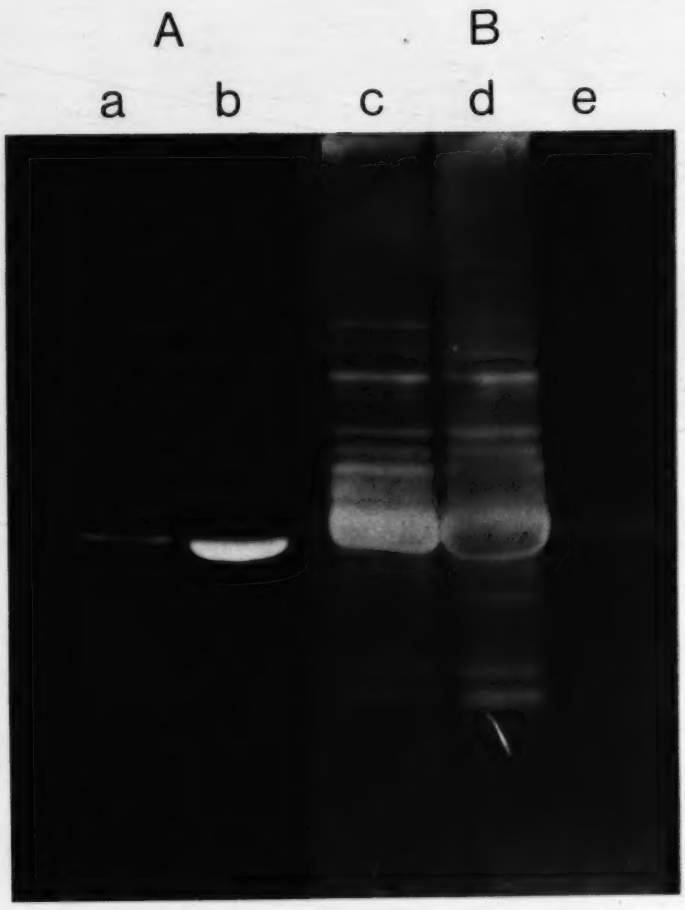
a, [^{3}H]-met-tRNA

b, [^{14}C]-formyl-[^{3}H]-met-tRNA

c, [^{3}H]-met-tRNA

d, [^{14}C]-formyl-[^{3}H]-met-tRNA

e, *E. coli* tRNA


A
B
a
b
c
d
e

and Rabinowitz, 1984). It is concluded that the [^{14}C]-formyl-labelled product was fmet-tRNA.

Once optimal conditions for met-tRNA transformylase activity were established, levels of enzyme activity were measured at several times during germination and growth. The data for pea and peanut enzyme activity are shown in Figure 19. Although the absolute values at a given day were not identical, the trends were the same in both species; activity rose to a maximum and subsequently declined. The pea enzyme reached a maximum at 4-days, whereas in peanut, this occurred at ca. 3-days.

Protein Synthesis in Isolated Pea Mitochondria

The presence of an active met-tRNA transformylase in pea and peanut mitochondria implies organelle protein synthesis occurred during germination and growth (Leaver and Gray, 1982). To examine this possibility in pea, incorporation of radioactive amino acids into cold TCA-precipitable material was studied in 4-day pea mitochondria.

The requirements for [^{3}H]-leucine (Table 9) and [^{35}S]-methionine incorporation (Table 10), were found to be very similar. A complete reaction system having no incubation period was used as the 'blank'. Radioactivity detected with this zero time control was subtracted from all other determinations. Incorporation was ADP-, malate- and Mg^{2+}-dependent. Chloramphenicol inhibited incorporation,

FIGURE 19

CHANGES IN THE ACTIVITY OF 10-FORMYL-H$_4$PTEGLU: MET-tRNA TRANSFORMYLASE IN MITOCHONDRIA ISOLATED FROM PEA AND PEANUT DURING EARLY GROWTH

The activity of the enzyme from pea (▲——▲) and peanut(●——●) is given as pmoles fmet-tRNA formed per 100 pmoles tRNA recovered/mg mitochondrial protein. Enzyme activity was proportional to protein concentration in all assays, and was determined from at least 3 protein levels, in duplicate, from one mitochondrial extraction. Each point represents the mean ± S.E.M. from at least 2 separate extractions.

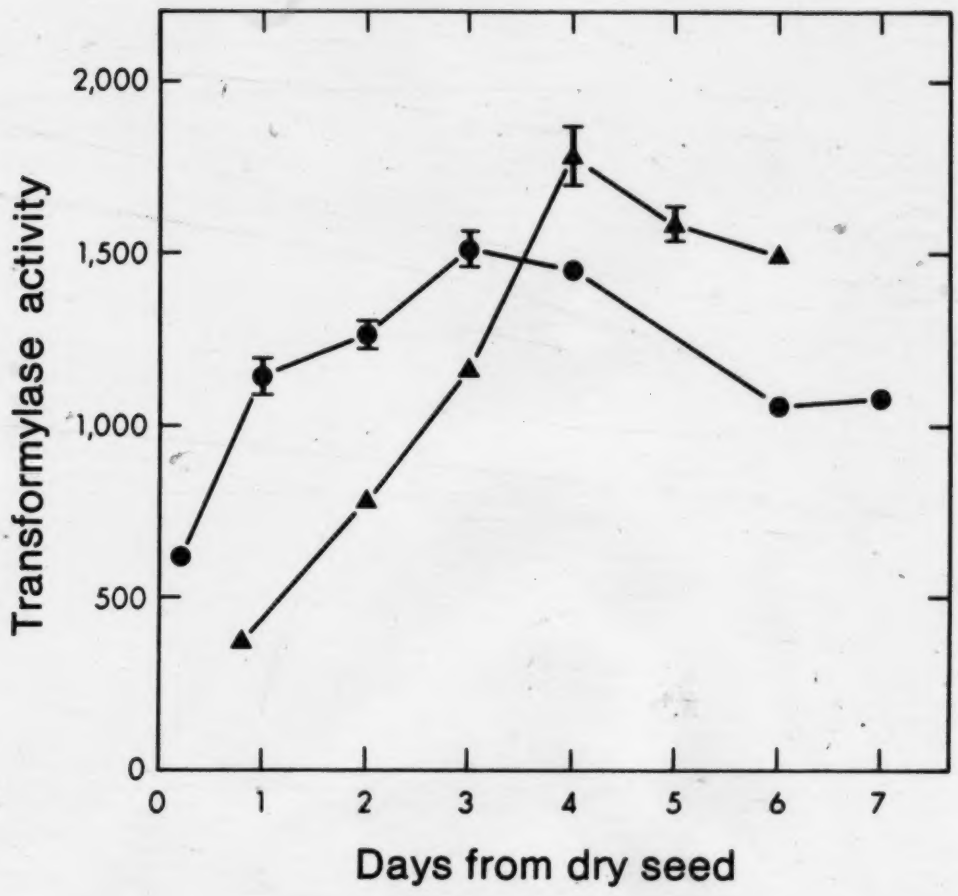

Transformylase activity
2,000
1,500
1,000
500
0
0
1
2
3
4
5
6
7
Days from dry seed

Table 9. Requirements for [4, 5-^{3}H]-leucine incorporation by isolated pea mitochondria.

Assay conditions	Incorporation*	%
complete system	2038	100
minus ADP	n.d.	0
minus ADP, plus ATP**	3913	192
minus Mg^{2+}	102	5
plus chloramphenicol (100 µg/ml)	734	36

* Incorporation is expressed as DPM (^{3}H)-leucine per mg mitochondrial protein, in a 30 minute assay.

**ATP concentration = 1 mM.

Table 10. Requirements for [^{35}S]-methionine incorporation by isolated pea mitochondria.

Assay Conditions	Incorporation*	%
complete system	19820	100
minus malate	7333	37
minus ADP	7135	36
minus ADP, plus ATP**	42415	214
plus chloramphenicol (100 µg/ml)	7730	39
plus cycloheximide (100 µg/ml)	17442	88

* Incorporation is expressed as DPM ^{35}S-methionine per mg mitochondrial protein, in a 45 minute assay. Mitochondria were from 4-day-old cotyledons.

**ATP concentration = 1mM.

whereas cycloheximide exhibited a much smaller effect. The omission of ADP, coupled to ATP addition, yielded values twice the controls. This finding was consistently observed, although the reason for this increase was not clear. It is possible that insufficient endogenous synthesis of ATP was occurring.

In the present incorporation system, ADP and malate were supplied as the energy source. ATP required was therefore synthesized endogenously. Since ATP synthesis is dependent on intact mitochondria, the effect of a 1 hour incubation on respiratory activity of isolated mitochondria was studied. The data (Table 11) showed that there was little decrease in this activity and therefore the incubation period did not seriously affect these organelles.

A time course of amino acid incorporation and the effect of protein concentration were routinely determined with each separate mitochondrial isolation. The data presented in Figure 20, show typical curves obtained for the above parameters. The incubation period was chosen as 45 minutes as incorporation was consistently linear for assays of this length. Values reported for incorporation (dpm/mg protein) were only those from the linear portion of the protein curve. Magnesium ion concentration exhibited a sharp optimum of 15 mM (Figure 21) and therefore this concentration was used in all assays.

In assays of organelle protein synthesis, the presence of bacteria can seriously affect the levels of incorporation

Table 11. Effect of incubation period on respiratory activity of isolated pea mitochondria.

	ADP/O	RCR
time zero	1.21	2.21
1 hour incubation	1.23	1.95

Mitochondria were isolated and respiratory parameters assessed as described in Materials and Methods. A sample of the final mitochondrial suspension was assessed immediately after isolation and 1 hour after incubation at 25°C. Data presented are means of three replicates.

Figure 20

EFFECT OF TIME AND PROTEIN ON INCORPORATION OF [^{3}H]-LEUCINE BY ISOLATED PEA MITOCHONDRIA

Incorporation was measured as described in Materials and Methods. In the time course (A) duplicate samples were removed at several intervals and treated as described. The effect of protein (B) was measured as a function of mitochondrial protein. Each point represents the average of two determinations. The bars represent the range in values.

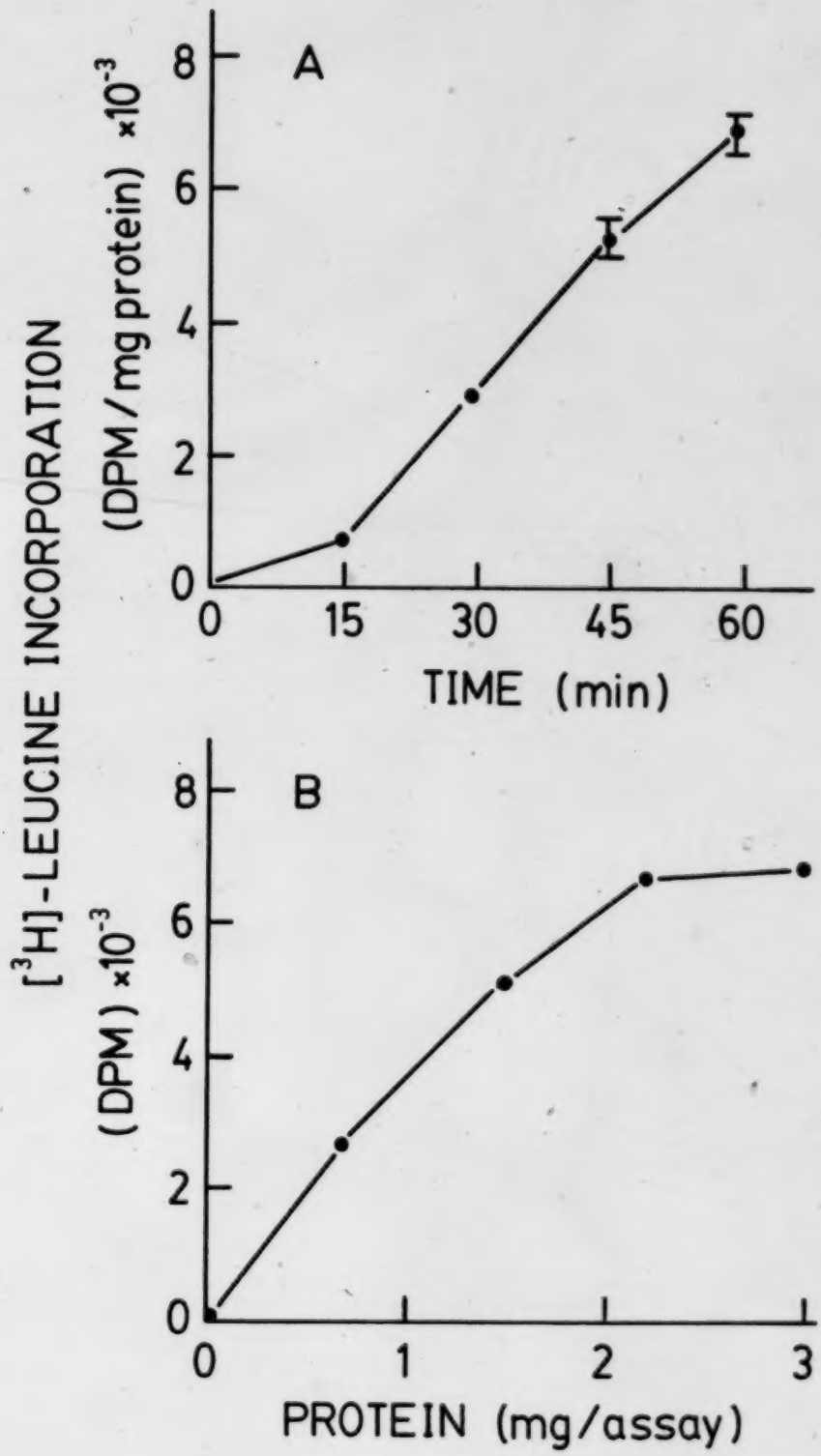
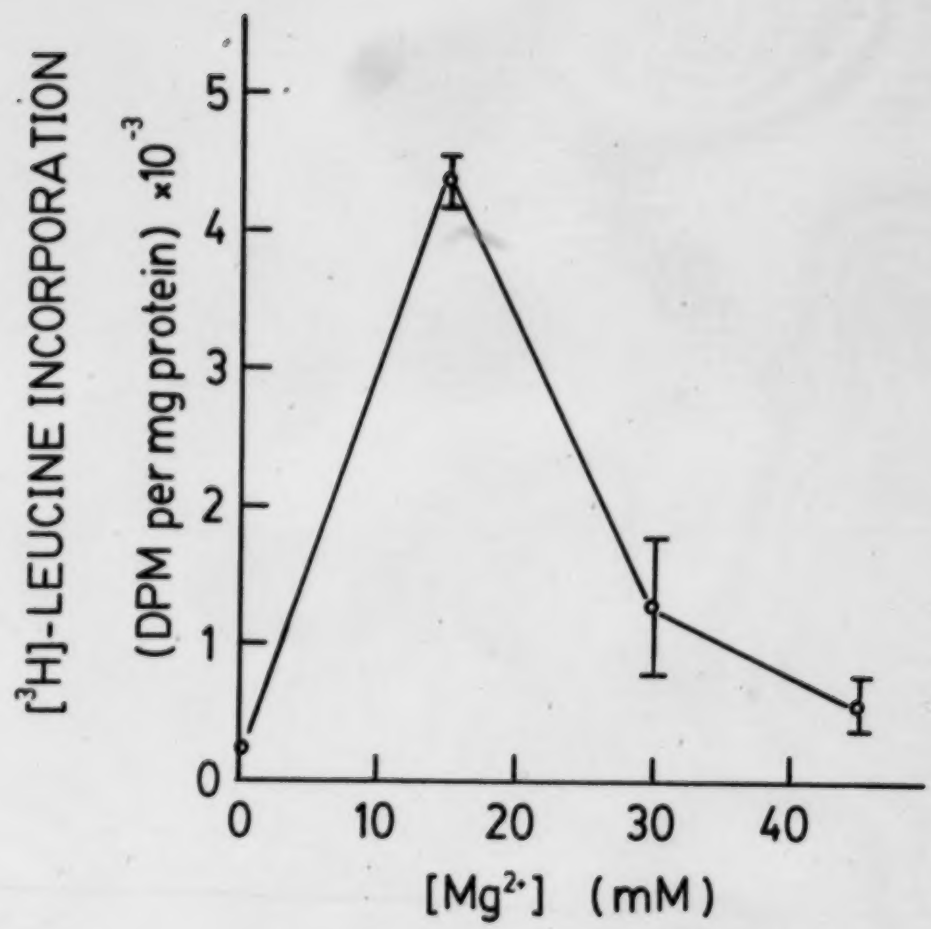

A
8
6
4
2
0
0
15
30
45
60
TIME (min)
[3H]-LEUCINE INCORPORATION
(DPM/mg protein) ×10-3
B
8
6
4
2
0
0
1
2
3
PROTEIN (mg/assay)
(DPM) ×10-3

Figure 21

EFFECT OF MAGNESIUM ION CONCENTRATION ON INCORPORATION OF [^{3}H]-LEUCINE BY ISOLATED MITOCHONDRIA

Incorporation was measured as described in Materials and Methods. Each point represents the average of two determinations. The bars indicate the range in values.


[3H]-LEUCINE INCORPORATION
(DPM per mg protein) ×10-3
0
1
2
3
4
5
0
10
20
30
40
[Mg2+] (mM)

detected (Forde et al., 1979). Therefore bacterial contamination was assessed for every mitochondrial isolation. This was done by incubation of mitochondria, in the complete reaction mixture, under conditions identical to the usual assay and plating aliquots of this on plate count agar. The levels of contaminant bacteria were about 2000 per assay or less. Previous studies by Forde et al. (1979), demonstrated that such levels would not make significant contributions to the observed rates of leucine incorporation. Data were discarded if higher bacterial levels were present.

Protein synthesis was also studied in isolated peanut mitochondria, but persistently high bacterial levels (10^5-10^6 per assay) resulted in poor reproducibility and the results are therefore not reported here. Several different treatments to remove bacteria were attempted, including washes with the detergent, Haemosol, but these met with little success. It is quite possible the oily nature of peanut seeds made removal of bacteria extremely difficult.

After conditions for optimal incorporation were determined, protein synthesis was examined in isolated pea mitochondria of two different ages. Amino acid incorporation in 4-day pea mitochondria was approximately 5-fold the level observed in mitochondria isolated from 16 hour-old cotyledons (Table 12).

Table 12. Incorporation of [4,5-^{3}H]-leucine by 16-hour and 4-day-old pea mitochondria.

	Incorporation (DPM/mg protein)
16 hour	881 (727 - 1124)
4 day	4662 (3600 - 4389)

Incorporation was as described in Materials and Methods. The values presented are the averages of replicates from 2 separate mitochondrial isolations of each age. Numbers in parentheses show the range in values.

IV. Discussion

The five areas presented in the Results section will now be discussed. Mitochondrial isolation and respiratory activity will be discussed together, whereas the remaining three topics are discussed separately. The final segment of this section of the thesis will be devoted to a discussion of possible relationships between folates, mitochondrial protein synthesis and the development of mitochondria during germination and growth of seeds.

Mitochondrial Isolation and Respiratory Activity

On the basis of widely accepted criteria, it is reasonable to conclude that the methods used in the present study resulted in the isolation of organelles that were essentially free of contamination by other cellular components (Tables 2 and 3). It is clear that, in general, the density gradient step significantly reduced the specific activities of alcohol dehydrogenase and catalase, in addition to reducing the total number of units of these enzymes. This step also significantly increased the specific activity of succinic dehydrogenase.

Previous studies have shown that, in rat liver, organelles other than mitochondria contain significant levels of folate derivatives (Shin et al., 1976). The presence of folates in peroxisomes has also been suggested for plants (Clandinin and Cossins, 1972). These authors isolated mitochondria from 4-day-old pea cotyledons using a

multi-step sucrose gradient. Although the protein bands in this gradient exhibited catalase activity and detectable folates, these derivatives were present at low levels; less than 10% of the folate present in the mitochondrial band. Thus, the low percentage of catalase units (ca. 5% of the total) revealed in the mitochondrial suspension of the present study would not significantly affect the measurement of mitochondrial folates even if some peroxisomal folate was present. Protein synthesis has not been demonstrated in peroxisomes (Beevers, 1979) and would therefore not affect determination of the levels of methionyl-tRNA transformylase activity or mitochondrial protein synthesis.

It is generally accepted that ADP/O and respiratory control ratios can be used as criteria for intactness of isolated mitochondria (Palmer, 1976; Quail, 1979; Tzagoloff, 1982). The ratios determined for mitochondria isolated from 4-day pea and peanut cotyledons (Tables 2 and 3) indicated some degree of integrity existed in these organelles. These findings, coupled with the data from the marker enzyme studies, clearly show that active mitochondria are obtainable with the present method.

The RC ratios in peanut are somewhat higher than in pea (compare Figures 3 and 4). This may be in part due to the presence of cyanide-insensitive respiration in pea (James and Spencer, 1979). These studies showed that levels of cyanide-insensitive respiration increased markedly during early growth and reached a peak at approximately 6 days. The

presence of these high levels would result in higher State 4 respiration rates and would therefore reduce the RC ratios, since these ratios are calculated from the ratio of State 3/State 4 respiration rates. Thus, it is possible that the pea RC ratios are minimal values, particularly after 4 days. To this author's knowledge no studies of cyanide-insensitive respiration have been performed using peanut, therfore possible effects of this type of respiration on the values reported for respiratory parameters in this species, are unknown.

Studies of ADP/O and respiratory control ratios of both species during the first 7 days of growth indicated that mitochondria isolated from these species had an increasing efficiency for coupled ADP phosphorylation. These data strongly suggest the mitochondria were initially poorly-developed. Organelles isolated after 3 to 4 days, displayed the properties exhibited by mitochondria that are fully-developed with respect to ultrastructure and biochemical capability. Thus, there appears to be a development of mitochondria in both species.

This contention is supported by several lines of evidence. As noted in the Introduction, ultrastructural studies of the pea cultivar, Homesteader, revealed that mitochondria isolated at 1 day showed poorly-developed cristae, whereas at 4 days mitochondria with well-developed cristae were evident (Solomos et al., 1972). These findings are consistent with ultrastructural examination of whole

cotyledons (Solomos et al., 1972). These authors also showed these changes were paralleled by increases in activity of key mitochondrial enzymes. Similar trends were observed in peanut (Morohashi et al., 1981). The presence of high levels of alcohol dehydrogenase in the Homesteader pea, during the first 2-3 days (Cossins, 1978), suggests that the mitochondria may have a reduced capacity for normal aerobic metabolism. Alcohol dehydrogenase activity is almost undetectable at the time when the mitochondria exhibit maximal rates of oxygen uptake, and high respiratory control ratios (Cossins et al., 1968; Solomos et al., 1972).

Studies of other cultivars of pea, have yielded analogous results. For example, in the Alaska cultivar, maximal ADP/O and respiratory control ratios were observed after 24-48 hours (Nawa and Asahi, 1971;1973a). Ultrastructural examination revealed fully-developed mitochondria after only 18 hours (Nawa and Asahi, 1973b). In Victory Freezer, well-developed organelles were evident after 2 days (Bain and Mercer, 1966). Therefore, although the timing is variable, the same trends are observed in all cultivars. The rates of oxygen uptake observed by the above authors, in addition to the present investigation, paralleled the changes in ADP/O and respiratory control ratios.

In the present study, the higher levels of cytochrome $a+a_3$ in pea mitochondria isolated after 3 days, compared to those of 16 hour organelles (Figure 5), suggest that the

observed increases in respiratory rates may be related, at least in part, to the higher levels of this component. This contention is supported by previous studies of pea where cytochrome oxidase activity increased markedly during this same period, to reach a peak at 4 days (Solomos et al., 1972). The activity at 3 days was 80% of the level at 4 days. Cytochrome difference spectra have been used by several authors to investigate the synthesis of cytochrome $a+a_3$ (Ashour et al., 1980; Sakano and Asahi, 1971). Difference spectra measured in mitochondria of blowfly flight muscle isolated at several times during a 13-day period, revealed increases in the amount of cytochrome $a+a_3$ (Ashour et at., 1980). As noted in the Introduction, mitochondria of newly-emerged flies underwent biogenesis, as shown by their high sensitivity to chloramphenicol. Injection of these flies with the inhibitor prevented the increase in cytochrome $a+a_3$ levels. It is interesting to note that this treatment also severely inhibited the normal increases in mitochondrial protein and rates of oxygen uptake. Respiratory control ratios also exhibited a 50% decrease, although ADP/O ratios showed little change.

Sakano and Asahi (1971a) measured difference spectra of mitochondria isolated from fresh sweet potato tuber and from tissue sliced and aged 20 hours. Ageing of the tissue resulted in significant increases in levels of the 'b' cytochromes (551,559 and 562) and cytochrome $a+a_3$. The effect of inhibitors of protein synthesis on these spectra

was not investigated, although chloramphenicol eliminated the increase in cytochrome oxidase activity observed in untreated controls. In view of the above studies, it is conceivable that the increased cytochrome levels observed in the present study reflected a synthesis *de novo* of these components. Confirmation of this would require a study similar to Dixon et al. (1980), who examined the changes in the levels of cytochrome oxidase of *Vicia faba* using an immunological approach.

Mitochondrial Folates of Pea and Peanut

The fundamental role of folates in cellular metabolism has prompted a considerable amount of research into the biochemistry of these compounds, particularly in animal systems (Blakley and Benkovic, 1984; Cossins, 1980). A majority of these studies have examined tissue of a specific age. Relatively few studies have examined folate metabolism from a developmental perspective. Previous work from this laboratory established that a net synthesis of folate derivatives occurred in pea cotyledons during germination and growth (Roos and Cossins, 1971). The data presented in Figure 6 confirms these earlier studies and extends them to include changes in the levels of polyglutamyl folates, during the same period. These conjugated derivatives, containing more than 3 glutamyl residues per molecule, made up at least 70% of the folate pool, after the first day. Initially, the pool consisted of only 38% polyglutamates.

These data are also consistent with recent studies of folylpolyglutamate synthetase in pea cotyledons (Cossins and Chan, 1984). These authors showed that synthetase activity rose rapidly during the first three days of germination and growth. This enzyme formed tetraglutamates in short assays, but longer incubations resulted in hexaglutamate formation. Derivatives with such chain lengths are poor substrates for *L. casei* (Tamura et al., 1972), so their presence in cotyledon samples is difficult to detect prior to conjugase treatment (Figure 6).

The levels of polyglutamates presented are likely minimal values. Although it is not evident in the data reported for free cotyledon folates (Figure 6), larger variation was encountered in assay of samples before conjugase treatment, than after. As the aliquot volume added to culture tubes was increased, the folate concentration (ng/ml of sample) also increased. This observation has been noted by other authors (Spronk, 1971; Tamura et al., 1972). This phenomenon referred to as 'positive drift' is routinely found during assay of samples that contain significant amounts of polyglutamates (Tamura et al., 1972). Although *L. casei* has a very low response to folates with a chain length greater than three, Tamura et al., (1972) obtained good evidence that they were utilized. This phenomenon results in an over-estimation of free folate levels and an underestimation of polyglutamates.

The nature of the folate pools in cotyledons was examined by differential microbiological assay (Figure 6). The low growth response of *P. acidilactici*, relative to *L. casei*, indicated that only a small percentage of the pool consisted of formyl derivatives and that the majority of the pool was 5-CH_3-H_4PteGlu. This finding is in agreement with Roos and Cossins (1971) who examined these folates by DEAE-cellulose chromatography, in addition to differential microbiological assay.

To this author's knowledge, no studies of folate derivatives in peanut have been performed. It was, therefore, of interest to determine if a situation similar to pea, existed in this species. An examination of Figure 7 shows this to be true; a large increase in folate levels was observed over the same time period. Although, inhibitors of folate biosynthesis were not employed in this study, it is conceivable a net biosynthesis of folate derivatives occurred. Polyglutamate and formyl folate levels, as percentages of the total pool, were comparable to pea, although the levels of folates, expressed on a protein basis were about 3-fold higher in peanut. It is clear that the polyglutamate levels would likely also be minimal values, for the same reason as in pea.

Measurement of pea and peanut mitochondrial folates during germination and growth demonstrated that total folate levels increased significantly during the first 3 to 4 days (Figures 8 and 9). These changes paralleled those of

cotyledonary folates (Figures 6 and 7). Examination of the nature of the mitochondrial pools using differential microbiological assay, revealed that a majority of the pool consisted of formyl folates. These findings were confirmed by DEAE-cellulose chromatography (Figures 10 and 12). The greater proportion of formyl derivatives in mitochondria compared to whole cotyledons, provides evidence for a discrete folate pool in these organelles. The presence of different pools in mitochondria and whole cotyledons, conceivably reflects differences in the nature of the metabolic reactions occurring in each compartment.

Compartmentation of folates has been observed in several mammalian species (Corrocher and Hoffbrand, 1972; Gawthorne and Smith, 1973; McClain and Bridges, 1970; McClain et al., 1975; Sankar et al., 1969; Shin et al., 1976 and Wang et al., 1967), in addition to that observed in plants (Clandinin and Cossins, 1972; Shah and Cossins, 1970; Cossins and Shah, 1972). The nature of the mammalian mitochondrial folate pools has also been examined. For example, as noted in the Introduction, rat and guinea pig liver mitochondria contain predominately 10-HCO-H_4PteGlu and some 5-CH_3-H_4PteGlu (Wang et al., 1967; Corrocher and Hoffbrand, 1972, resp.). Mitochondria of Swiss mouse brain, however, contain only 5-CH_3-H_4PteGlu derivatives (McClain and Bridgers, 1970; McClain et al., 1975). The somewhat atypical nature of this finding led these authors to suggest it was due to a high rate of synthesis of biogenic amines, a

process that requires 5-CH_3-H,PteGlu. This latter folate predominates the folate pool of mammalian livers and the blood of several vertebrate species (Cossins, 1984). Therefore, the nature of pea and peanut mitochondrial pools is quite similar to pools of other unrelated species. It is interesting to note that total the folate levels of pea and peanut (33 and 43 ng/mg mitochondrial protein, resp.) are roughly comparable to the levels of these compounds in rat liver mitochondria (78 ng/mg, Shin et al., 1976).

As a result of the extraction method, the whole cotyledon pool will include mitochondrial folates. The presence of very low levels of formyl derivatives in the former, strongly suggests that the mitochondrial pool makes only a small contribution to the total folate pool of the cotyledon. In this regard, previous work has calculated that the mitochondrial pool accounts for only 17% of the total folates found in pea cotyledons (Clandinin and Cossins, 1972). This probably represents a minimal value, as folates will undoubtebly be lost during mitochondrial extraction (Clandinin and Cossins, 1972). These authors presented evidence that methyl derivatives were more tightly bound in mitochondria than formyl derivatives. This looser association may allow greater leaching of formyl derivatives and therefore, these compounds may actually make up an even greater proportion of the endogenous pool than revealed by the differential microbiological assays of the present work (Figures 8 and 9).

The increased folate levels observed in mitochondria during germination and early growth (Figures 8 and 9) could be due to synthesis *de novo* of folates, in the organelle, or alternatively to import of folates from the cytoplasm. Pea mitochondria contain enzymes of folate biosynthesis (Ikeda and Iwai, 1970, 1975; Okinaka and Iwai, 1970), but little is known about folate transport in plants. It is noteworthy that radioactive folylmonoglutamates are taken up by isolated mitochondria (Clandinin and Cossins, 1972). So the cytoplasm could conceivably supply some folate to the mitochondria. The available evidence (Cossins, 1980) also suggests that one-carbon (C-1) units required for generation of formyl and methyl folates are most likely supplied by serine and glycine arising from protein degradation. These units would be required to replace those used in folate-dependent reactions. For example, previous studies of folate metabolism in carrot disks revealed that serine contributed to the synthesis of the methyl group of methionine (Fedec and Cossins, 1976) and the ability of pea mitochondria to generate C-1 units from serine and glycine has been reported (Clandinin and Cossins, 1972). It is clear, therefore, in pea, mechanisms exist that could account for the observed increases in mitochondrial folates. The present data do not allow a choice between the alternatives outlined above, although it is conceivable that both could contribute to the changes observed during germination and growth.

Microbiological assay of pea and peanut mitochondrial folate pools, before and after conjugase treatment, revealed the presence of significant levels of folylpolyglutamates (Figures 8 and 9). This finding is not unexpected as the majority of species have native folates that exist as polyglutamates of variable chain length (Kisliuk, 1981; McGuire and Bertino, 1981; McGuire and Coward, 1984). This is also consistent with the general observation that folylpolyglutamate synthetases have been found in all tissues studied to date (Cichowicz et al., 1981; McGuire and Coward, 1984). Studies of the subcellular localization of these enzymes have demonstrated their presence in the mitochondria of *Neurospora* and rat liver (Cossins and Chan, 1983; McGuire et al., 1979, resp.). The absence of detectable activity in pea mitochondria (Cossins and Chan, 1984), could be the result of the use of an inappropriate substrate, or suboptimal assay conditions. It is known that folylpolyglutamate synthetases can be quite specific with respect to the glutamate chain length of the folate derivative used as a substrate, in addition to its oxidation level (McGuire and Coward, 1984). The presence of polyglutamates in pea and peanut suggests that the synthetase is present in these organelles, because transport of polyglutamates into mitochondria, is not likely (McGuire and Coward, 1984).

The term 'formyl folates' usually refers to 5-HCO- and 10-HCO-H$_4$PteGlu, collectively. As indicated in the Results,

the presence of 5-HCO-H$_4$PteGlu may be an artifact, since the levels of this derivative could be accounted for by isomerization of a portion of the 10-HCO-H$_4$PteGlu pool (Figures 10 and 11). This finding is not unexpected since the physiological role of 5-HCO-H$_4$PteGlu has yet to be established (MacKenzie, 1984). Although this compound can be utilized by cells, its status as a naturally-occurring folate is not clear (MacKenzie, 1984). Despite this, the rapid utilization of 5-HCO-H$_4$PteGlu by replicating cells (MacKenzie, 1984), implies that enzymes must exist for conversion of this folate into native derivatives. Whether or not such enzymes are specific for 5-HCO-H$_4$PteGlu, is not known.

In the present investigation, because the levels of 5-HCO-H$_4$PteGlu can be accounted for by isomerization, and also that other derivatives that cause a growth response in *P. acidilactici*, are not detectable, it is logical to assume that the levels of folates determined using this organism, represent levels of 10-HCO-H$_4$PteGlu. On the basis of the present data, the possibility that 5-HCO-H$_4$PteGlu is present at low levels and has some physiological role, cannot be totally excluded, although it is clear that this derivative does not constitute a significant part of the endogenous pool.

Activity of 10-formyl-H_4PteGlu: met-tRNA Transformylase

Methionyl-tRNA transformylase has been demonstrated in several prokaryotes, as well as the organelles of higher organisms (for a review, see Staben and Rabinowitz, 1984). This enzyme has been shown to be present in all systems studied to date and is therefore probably ubiquitous. Previous work has been confined to establishing the presence of the enzyme in a particular system, and sometimes its purification and characterization (Staben and Rabinowitz, 1984). To this author's knowledge, none of these earlier studies examined the activity of the enzyme in a developing system. Therefore, the present study investigated the activity of the transformylase in mitochondria of pea and peanut, during germination and growth.

In order to accurately compare enzyme activities of different tissues, conditions for optimal assay were established for both species (Tables 6 and 7; Figures 13-17). These studies included the effect of varying the concentrations of 10-HCO-H_4PteGlu$_1$, and met-tRNA (Figures 14 and 16). There has been only one study to date where a polyglutamate substrate was used in a transformylase assay (Samuel and Rabinowitz, 1974). The Michaelis constants determined for 10-HCO-H_4PteGlu$_1$ and 10-HCO-H_4PteGlu$_3$, were not significantly different. This result is surprising in view of reports for other folate-dependent enzymes where polyglutamyl substrates are often the preferred substrate (McGuire and Coward, 1984). For example, yeast

formyltetrahydrofolate synthetase has significantly greater affinity for polyglutamate substrates. Accordingly, the Michealis constant decreases sharply from 265 uM with one glutamate residue, to 0.24 uM, when the substrate had five residues (Rabinowitz, 1983). It is also clear that the level of glutamylation has a very significant effect on other enyzmes of folate metabolism (McGuire and Coward, 1984). The folate specificity of the plant transformylase, clearly warrants further study, particularly in light of the finding (Figures 8 and 9) that 10-HCO-H$_4$PteGlu polyglutamates occur in plant mitochondria.

E. coli was the source of tRNA used in the present study and met-tRNA was formed using an *E. coli* met-tRNA synthetase (Table 4). Studies of the transformylase from *Euglena* chloroplasts showed that this enzyme can formylate *Euglena* or *E. coli* met-tRNA with equal affinity (Crosti et al., 1977). For this reason, and because of its availability, *E. coli* tRNA was used in the present study. Halbreich and Rabinowitz (1971) have shown that the yeast mitochondrial transformylase prefers the homologous met-tRNA over *E. coli* tRNA. It would, therefore, be interesting to determine if a similar preference is exhibited by higher plant mitochondria. It is unlikely that the potential stimulatory effect of the homologous tRNA or a polyglutamyl substrate would change during the period of study. Therefore, although the values reported for the levels of transformylase activity (Figure 19) may be minimal values,

it is clear that the trends shown by these data are valid.

Transformylase activity rose to maximal levels after 4 days in pea and 3-4 days in peanut, and subsequently declined (Figure 19). It is interesting that the levels of 10-HCO-H_4PteGlu in both species closely paralleled changes in transformylase activity. This can easily be seen if mitochondrial folate levels and enzyme activity are re-plotted on the same figure for each species (Figure 22). In pea, 10-HCO-H_4PteGlu levels remained relatively constant after 4 days, whereas the total folate level continued to increase. In peanut, there was a much sharper drop in 10-HCO-H_4PteGlu levels and a larger increase in total folates. In both species, the increase in total folates is due to rising levels of 5-CH_3-H_4PteGlu. This derivative has been shown to inhibit the transformylase of *Euglena* chloroplasts (Gambini et al., 1980). Although it is tempting to conclude that the increasing levels of 5-CH_3-H_4PteGlu were at least partly responsible for the drop in transformylase activity, there would appear to be little physiological advantage for such a control mechanism.

Protein Synthesis in Isolated Pea Mitochondria

The presence of an active met-tRNA transformylase implies that the mitochondria of both species were actively synthesizing protein. This possibility was examined further in pea, in a study of amino acid incorporation into mitochondrial protein. After the requirements for

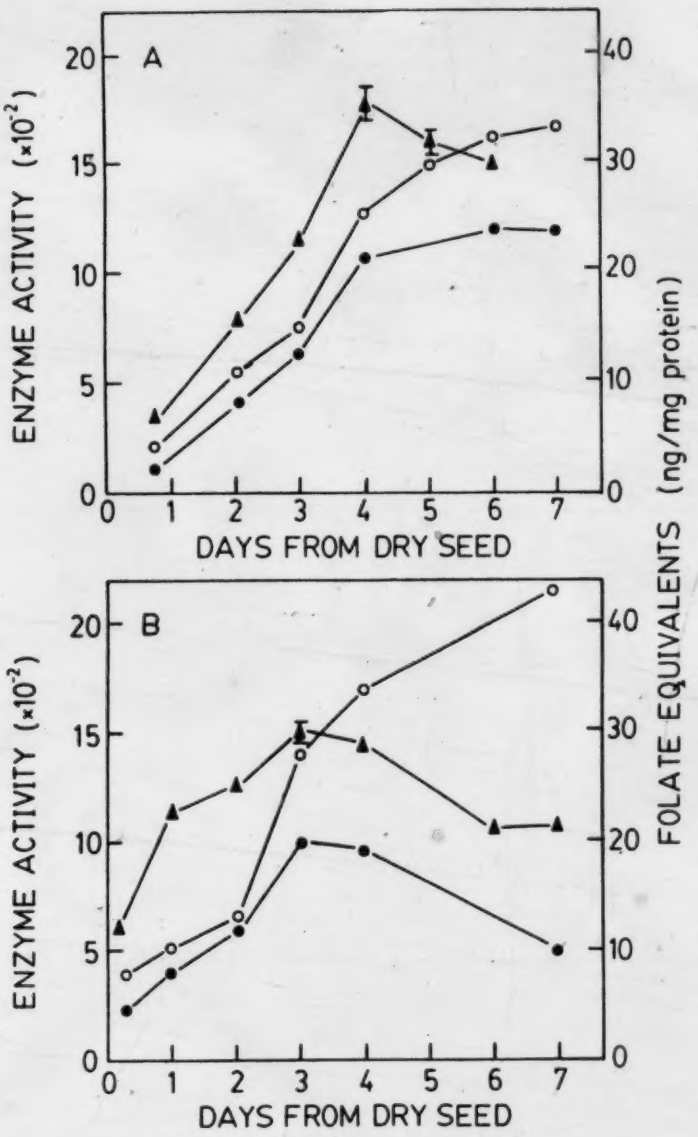


Figure 22. **COMPARISON OF METHIONYL-tRNA TRANSFORMYLASE ACTIVITY WITH MITOCHONDRIAL FOLATE LEVELS**

A, pea; B, peanut. Transformylase activity is expressed as in Figure 19. Symbols, (▲——▲) transformylase activity, (o——o) *L. casei* and (●——●) *P. acidilactici* assay after conjugase treatment.

incorporation were established (Tables 9-11; Figures 20 and 21), rates of incorporation were determined for mitochondria of two ages. The 5-fold increase in the rate of incorporation of ^{3}H-leucine into protein (Table 12), is consistent with the levels of met-tRNA transformylase activity, which exhibited a 4-fold increase during the same period of time (Figure 19).

As noted in the Introduction, organelle protein synthesis has many similarities in common with bacterial protein synthesis. Therefore the presence of bacteria in assays of amino acid incorporation may seriously affect the results. In the present investigation, incorporation was shown to be ADP-dependent (Tables 9 and 10). If bacterial levels were high (10^5 or greater), absence of ADP had no effect, and, in addition, much higher levels of incorporation were observed (data not shown), demonstrating that, in the present system, bacterial contamination must be monitored. If the numbers of bacteria were about 2000 or less per assay, ADP-dependence was clearly evident. Although the plate count assays used to enumerate bacteria would not likely detect all bacteria present, the 1000-fold reduction in levels as a result of ethanol and hydrogen peroxide washes of the cotyledons, clearly shows that these treatments were effective in reducing bacterial contamination. This finding, in conjunction with the observed ADP- and malate-dependence, suggests that the observed rates of incorporation are due only to

mitochondrial protein synthesis (Table 12).

The present data concerning amino acid incorporation are also in agreement with a recent report that demonstrated the occurrence of mitochondrial protein synthesis in cotyledons of *Vicia faba* during germination and growth (Dixon et al., 1980). These authors showed a 10-fold increase in the rate of amino acid incorporation during the first 4 days after the onset of imbibition. Immunoprecipitation of mitochondrial proteins with cytochrome c oxidase subunit I antibodies indicated that this enzyme was synthesized at increasing rates during the period of study. Furthermore, changes in the rate of oxygen uptake closely paralleled those of amino acid incorporation.

Possible Relationships between Folate Pools, Transformylase Activity and the Development of Mitochondria

In the present study, mitochondrial development in pea and peanut appears to be very similar. In both species, an active transformylase could be detected (Figure 19) and the levels of 10-HCO-H$_4$PteGlu, closely parallel changes in enzyme activity (Figure 22). Maximal rates of the transformylase occur at the same time as maximal respiratory activity (compare Figures 3, 4 and 22). In pea, the 5-fold increase in the rate of amino acid incorporation from 16 hours to 4 days is consistent with data on the transformylase. The present data, therefore, show that protein synthesis is occurring in mitochondria of both

species during germination and early growth. These data also strongly suggest that the observed increases in respiratory activity are at least partly derived from this synthesis of mitochondrial proteins.

Rapid increases in respiratory activity have been observed, upon imbibition, in a number of species (for reviews, see Bewley and Black, 1978; Pradet, 1982). The period of time required for attainment of maximal rates is species-dependent and varies widely. For example, in mitochondria isolated from germinating spores of *Botryodiplodia*, cytochrome oxidase activity reached a plateau after only 3.5 hours (Brambl,1980). Mitochondria from *Typha* pollen appear to have no lag period at all, and exhibit maximal rates of oxygen uptake immediately after rehydration of pollen grains (Hoekstra and van Roekel, 1983). This has also been demonstrated for pollen grains from other species, where pollen tube emergence begins within minutes of rehydration (Hoekstra, 1979). Maximal rates of oxygen uptake in germinating and growing seeds are reached after a somewhat longer period of time. For example, as noted in the Introduction, the pea cultivar, Alaska, attains maximal respiratory activity within 1-2 days, whereas in the Homesteader cultivar, this does not occur until 4 days (Nawa and Asahi, 1971,1973a; Solomos et al., 1972, resp.).

Several modes of mitochondrial development appear to be operating in the above species. In pollen grains,

mitochondria appear to be intact in the dry grains, since reappearance of maximal rates of oxygen uptake is so rapid and pollen tube growth starts within minutes of rehydration (Hoekstra and van Roekel, 1983). As expected, inhibitors of mitochondrial protein synthesis had no effect on respiratory activity (Hoekstra, 1979). Thus, mitochondria in this species appear to be fully-developed at all stages. In *Botryodiplodia*, chloramphenicol applied to germinating spores appeared to have no effect on increasing cytochrome oxidase activity until 2 hours, just prior to germ tube emergence (Brambl, 1980). It is at this time that a rapid increase in cytochrome oxidase is observed. It was also demonstrated that all subunits of cytochrome oxidase (mitochondrially and cytoplasmically encoded) were synthesized at this time (Brambl, 1980). The author concluded that in the initial stages of germination, mitochondrial protein synthesis was not required and the increases in cytochrome oxidase activity were due to reassembly of subunits already present. He also concluded that the rapid increase in activity after 2 hours was at least partly due to synthesis *de novo* of cytochrome oxidase. Therefore, in *Botryodiplodia* , mitochondrial development is clearly evident, and that this development occurs via reassembly of pre-existing components, in addition to their synthesis *de novo* .

Detailed studies of mitochondrial development during seed germination and early growth have been performed in

only a few legume species (Bewley and Black, 1978; Dixon et al., 1980). It has been proposed that in the Leguminosae, two modes of mitochondrial development occur. In pea, mitochondrial protein synthesis is not essential and development occurs via improvement of pre-existing organelles. In peanut, development occurs via mitochondrial biogenesis. The recent study of Dixon et al. (1980) of *V. faba*, suggests that development in this species is of the 'peanut type'.

The data for peanut, in the present study, are in agreement with earlier reports of Breidenbach et al. (1966, 1967), that suggested that the number of mitochondria increased during germination and early growth, a process requiring the synthesis of mitochondrial proteins. The present data also support more recent studies that demonstrated the occurrence of biogenesis in this species (Morohashi et al., 1981a and b). The contention that the protein synthesis shown in pea mitochondria is at least partly responsible for the observed increases in respiratory activity appears, however, to be in contrast to some previous reports that concluded that this process was not required (Morohashi,1980; Morohashi and Bewley, 1980; Nawa and Asahi, 1973b). The present data, are however, supported by earlier studies of the Homesteader variety (Malhotra et al., 1973; Malhotra and Spencer, 1973). These authors concluded that mitochondrial protein synthesis did play a role in the structural development of pea cotyledon

mitochondria.

Nawa and Asahi (1973), showed that [^{14}C]-leucine was incorporated into mitochondrial protein, during the first 18 hours of germination if seeds were allowed to imbibe this amino acid, with and without inhibitors. Cycloheximide (CHI) inhibited this incorporation by about 90%, whereas chloramphenicol (CAP) caused only a 12% decrease, compared to controls. These authors concluded that, since only CHI significantly inhibited incorporation, only cytoplasmic protein synthesis was occurring. The effect of these inhibitors on increases in respiratory control ratios, and cytochrome oxidase and malate dehydrogenase activities, was also studied (data not shown in the paper), and neither inhibitor blocked the increases. These authors concluded that synthesis of mitochondrial proteins, in the cytoplasm or mitochondrion, was not essential for mitochondrial development.

It has been demonstrated that mammalian and fungal mitochondria synthesize only 5-10% of the protein in these organelles (Tzagoloff, 1982). The same trend appears to be present in mitochondria of higher plants (Leaver and Gray, 1982). Therefore, in *in vivo* experiments, where CAP is used, cytoplasmic protein synthesis would continue, with labelled proteins being exported to the mitochondria. Because of the very large contribution of the cytoplasm, protein synthesis in the presence of CAP would be expected to exhibit only small decreases in incorporation. The 12% inhibition

observed by Nawa and Asahi (1973) may, therefore, be significant.

The lack of effect of CHI or CAP on cytochrome oxidase, malate dehydrogenase, and respiratory control ratios, is somewhat difficult to interpret. The levels of CAP used by these authors are one-sixth the concentrations used by Asahi's group in their study of mitochondrial biogenesis in sweet potato (Sakano and Asahi, 1971a and b). In the latter work, these workers presented evidence that CAP eliminated the increase observed in cytochrome oxidase activity. It is possible that in the pea study, (Nawa and Asahi, 1973), insufficient inhibitor levels were used. More recent studies have shown that, in blowfly flight muscle, the concentration of the inhibitor significantly affected the inhibition observed (Ashour et al., 1980).

Malate dehydrogenase may not be a suitable enzyme to study in an investigation of this nature. Although this enzyme is present in mitochondria, it is also found in other organelles, most notably peroxisomes (Quail, 1979). Nawa and Asahi (1973), used a crude mitochondrial preparation in their inhibitor studies. In the present investigation, significant peroxisomal contamination was evident prior to sucrose density gradient centrifugation (Tables 2 and 3). The possibility of peroxisomal contamination therefore, makes the data concerning malate dehydrogenase difficult to interpret, since it is not clear which enzyme is being examined.

Maximal respiratory rates in the Alaska cultivar are not reached until 1-2 days (Nawa and Asahi, 1971; Morohashi, 1980). It would be interesting to study the effects of inhibitors of protein synthesis for longer periods during germination. Perhaps mitochondrial protein synthesis would become more significant at later stages, similar to the situation in *Botryodiplodia* (Brambl, 1980). In more recent immunological studies, Asahi's group has shown that cytochrome oxidase can be detected within the first 24 hours (Matsuoka and Asahi, 1983). Thus, it is possible that mitochondrial development occurs via reassembly of pre-existing components, as these authors contend. These findings are consistent with ultrastructural studies that showed well-developed mitochondria by 18 hours (Nawa and Asahi, 1973b), and the studies of mitochondrial development in germinating pollen grains (Hoekstra and van Roekel, 1983). However, a significant contribution of mitochondrial protein synthesis to development cannot be excluded on the basis of available evidence. Therefore, mitochondrial development may require a combination of reassembly and protein synthesis.

Morohashi (1980), in another study of the Alaska pea, showed that a 24 hour treatment of seeds with CHI, during imbibition, resulted in a 25% decrease in amino acid incorporation into soluble protein extracted with 10% TCA, from a 3000 g supernatant. This author also showed that the same concentration of inhibitor did not affect respiratory

rates or respiratory control ratios, although CHI concentrations 4-fold higher, resulted in a 20% inhibition of respiratory rates, but had no effect on respiratory control ratios. Similar results were obtained for mitochondria isolated after 18 hours (Morohashi and Bewley, 1980). As the respiratory rates observed by these authors were still increasing at 24 hours, it would be interesting to study the effects of inhibitors over longer periods of time. An examination of the effects of these inhibitors on mitochondria isolated from actively growing portions of the seedling, would also be useful. Active cell division occurs in these regions, as would mitochondrial biogenesis. If the concentration of inhibitors used in the previous studies were able to inhibit mitochondrial development in actively growing regions, then this would help to establish that these concentrations were effective.

Research similar to the studies of the Alaska pea, has been performed in the Homesteader cultivar (Malhotra and Spencer, 1973; Malhotra et al., 1973). In the latter studies, dry seeds were also allowed to imbibe solutions of inhibitors and labelled amino acid. Mitochondria were isolated after 3 and 4 days. Malhotra et al. (1973), showed that CHI caused a large (86%) decrease in incorporation, whereas CAP treatment resulted in only a 25% decrease. Erythromycin, also an inhibitor of mitochondrial protein synthesis, caused a 30% decrease. Initially, three populations of mitochondria exist in pea cotyledons, each

having a distinct density (Malhotra and Spencer, 1973). By 4 days, a single population is evident. As germination and subsequent growth proceeds, the two heavier populations disappear. Treatment of cotyledons with CAP prevents this disappearance, suggesting that mitochondrial protein synthesis is required for the development of these two populations (Malhotra and Spencer, 1973). Inhibitor treatment, however, did not affect respiratory rates, respiratory control ratios or ADP/O ratios.

The observation that respiratory activity in the Alaska cultivar reaches maximal levels 2-3 days before those in the Homesteader cultivar, suggests that differences exist in mitochondrial development in the two cultivars. The more rapid development in the former may result more from reactivation of pre-existing organelles rather than via biogenesis. Protein synthesis in the latter may have a more significant role. The lack of an effect of inhibitors on respiratory parameters, would suggest that any protein synthesis observed, is not essential for development of this activity. However, negative results can be sometimes be misleading. It is possible that measurements of protein synthesis are more sensitive to the inhibitors than respiratory parameters. For example, if the inhibitors do not penetrate to all the mitochondria present in the cotyledons, then it is conceivable that the unaffected organelles would remain active. There would be a greater demand for ATP synthesis in the presence of inhibitors, and

perhaps this could be met by increased activity in the unaffected organelles.

Previous studies of liver regeneration in rats have shown that the number of fully equipped respiratory chains must be reduced, by prevention of mitochondrial protein turnover by oxytetracycline, to approximately 10% of control values before impairment of regeneration occurs (van den Bogert et al., 1983). These authors have described this tissue as having a large reserve capacity for ATP generation. If such a capacity exists in cotyledons, then it may be difficult to properly assess effects of inhibitors unless a range of concentrations are tested. This study also examined the effect of different levels of oxytetracycline, and showed that little effect was observed at serum concentrations of 5-20 ug/ml, but significant inhibition was evident at concentrations of 21-25 ug/ml. These findings again illustrate the importance of establishing an effective inhibitor concentration.

The lack of significant difference during germination and early growth between pea and peanut, in the activities of the transformylase, levels of folates and respiratory activity strongly suggest that mitochondrial development in the two species is similar. More research is required in order to resolve the apparent differences between the findings reported for pea in the present data and previous studies.

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